British Medical Bulletin

Chromatography

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BOOK REVIEWS

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Scientific Editor: *C. E. Dent*

Chromatography

The aim of this number of the Bulletin is to give a general idea of the scope of chromatography, and of the ways in which it may be of service to medicine directly and indirectly. The literature of the subject has already become so large that it can no longer be followed in detail. It is evident that the main contribution of chromatography to medicine will be indirect, from the help that it gives to biochemistry as a whole, rather than its direct application to clinical research. But the pioneer work of C. E. Dent on amino-acid metabolism has shown how fruitful a direct application may be, and, without doubt, in time the number of these will increase greatly. It is probably true as a rough generalization that where measurement of a single substance

suffices a number of methods may be competitive, but that where information is required on a considerable number of related substances, chromatography, or the somewhat similar electrophoretic methods, will be supreme.

The increase in the use of chromatographic methods in biochemistry in the last decade has been spectacular. At the present time, inspection of any biochemical periodical will show that chromatography has become accepted as one of the standard methods of analysis and separation. It is indeed remarkable that it has proved possible to apply chromatographic methods to almost all the classes of substances with which biochemistry is concerned. In this number of the Bulletin, articles will be found covering the principal groups of substances of medical interest, inevitably a trifle arbitrarily selected. Unfortunately lack of space has precluded discussion of the related techniques of counter-current distribution and paper electrophoresis.

The aim of chromatography is to separate substances, for analytical or for preparative purposes. In comparison with the classical methods of the organic chemist it shows to particular advantage in the separation of very similar molecules. It is usually desirable to pre-treat mixtures to be subjected to chromatography, to remove components with widely different properties; for, in general, a chromatogram will take only a small load of material. Conversely there is practically no lower limit to the amount of substance which may be separated; it is the sensitivity of the means of detection which determines how little material may be used.

The recent development of the chromatogram has indeed gone hand in hand with the improvement or invention of new means of detection. An astonishing range of substances can now be detected on paper chromatograms by a remarkable array of methods. With or without chemical pre-treatment, absorption of visible, of ultra-violet or of infra-red radiation, fluorescence or the quenching of fluorescence, surface tension changes, and radioactivity, have all been used for detection by physical means. Promotion or inhibition of the growth of micro-organisms and various physiological reactions have been used for detection by biological means. Sensitive detectors are also available for gas–liquid chromatography and rapid development of the use of this method can now be expected.

By using displacement development instead of elution development, the amount of material that a chromatogram will handle is enormously increased, with some loss of resolving power. In this form the chromatogram is adapted for initial concentration and separation for preparative purposes. Gradient elution methods are intermediate in both the quantities handled and in the resolution obtained.

It is reasonable to expect that further new methods will continue to appear at frequent intervals. Each new method, particularly in so far as it depends upon different properties in the substances to be separated, increases the powers of the existing methods to deal with intractable mixtures. Perhaps nothing illustrates better the progress in methods made in the last fifteen years, than the field of protein chemistry. In 1938 the number of different kinds of amino acids present in any protein was not known with certainty, and an approximate amino-acid analysis of a single protein was a major undertaking. Today, thanks to F. Sanger, we know in detail which amino acid is linked to which in insulin. The total amount of insulin which he used in his determination of the structure is comparable with the amount that was once required for a single approximate amino-acid analysis.

Chromatography has afforded a ready means of performing multiple qualitative or semi-quantitative analyses and, with more expenditure of effort, quantitative analyses. It has also permitted the isolation of a number of substances which would have been extremely difficult to separate by older methods. This has, I believe, exerted a favourable effect on the mental attitudes of those engaged in biochemical research, so that there has been less reliance on long trains of argument verifiable only at the last stage, and a greater readiness to attempt a check of assumptions as early as possible.

The writer of an introduction is in a strong position to criticize others, as he is not expected to produce chapter and verse, which may at times be difficult and at others embarrassing. I have the "clinical impression" that my medically trained colleagues seldom feel the need to verify each link of their often protracted arguments. I hope that chromatography may ultimately play some part in the process, which I believe to be wholly desirable, of the conversion of medicine from an art to a science.

A. J. P. Martin

We wish to thank Dr A. J. P. Martin for having acted as Chairman of the committee that planned this number of the Bulletin, and for all the valuable advice and help he has given us.

Notes on Contributors

Dr I. E. Bush was at the University of Cambridge from 1946 to 1949 (Natural Sciences Tripos (Parts I and II)). He was awarded a Medical Research Council Research Scholarship and from 1949 to 1950 he worked at the Physiological Laboratory, Cambridge, and from 1950 to 1952 at the National Institute for Medical Research, London. During these years he worked on paper chromatographic methods for steroids and on the study of adrenocortical secretions in mammals, for his Ph.D. (Cantab.) degree which he was awarded in 1953. From 1952 to 1953 Dr Bush was granted a Commonwealth Fellowship in the USA, where he studied adrenal hormones at the University of Utah and later at the Huntington Laboratory, Massachusetts General Hospital, Boston. He is now concerned with part-time research on steroid hormones in man, while completing a medical degree at St Mary's Hospital, London.

Dr P. N. Campbell was an assistant lecturer in the Department of Biochemistry, University College, London, from 1947 to 1949 where, with Professor F. G. Young, he studied the biosynthesis of glycogen and especially the metabolism of 3-methyl glucose. He then took an appointment at the National Institute for Medical Research, London, where, together with Dr T. S. Work and Sir Edward Mellanby, he worked on the isolation of the toxic factor from agenized flour (*Biochem. J.* 1951, **48**, 106). In collaboration with Dr Work, he has since made various studies of the biosynthesis of proteins. In an initial chromatographic investigation of the peptides occurring in liver extracts, the presence of free glycerylphosphorylethanolamine was discovered (see *Biochem. J.* 1952, **50**, 449). More recently the work has involved a study of the biosynthesis of milk proteins in the rabbit and the goat (see *Biochem. J.* 1952, **52**, 217). Since January 1954, Dr Campbell has held an appointment at the Courtauld Institute of Biochemistry, Middlesex Hospital, London, where he is studying problems concerned with the biosynthesis of proteins in neoplastic tissue.

Professor A. C. Chibnall is a Fellow of Clare College, Cambridge. He began research in plant biochemistry at the Imperial College of Science and Technology, London, in 1920, and from 1923 to 1925 was a Research Fellow at Yale University, where he worked with the protein chemist, Dr T. B. Osborne. From 1925, until his appointment to the Chair of Biochemistry at the Imperial College in 1929, he was an honorary assistant in biochemistry at University College, London. In 1943 he was made Professor Emeritus of the Imperial College on his transfer to Cambridge as Sir William Dunn Professor of Biochemistry, a post from which he resigned in 1949. He was made a Fellow of the Royal Society in 1937 and was appointed Bakerian Lecturer in 1942. He gave the Silliman Lectures at Yale University in 1938, and was President of the First International Congress of Biochemistry at Cambridge in 1949. He is the author of *Protein metabolism in the plant* (1939) and of papers in scientific journals on proteins, waxes, and various aspects of plant metabolism.

Dr R. Consden, who is a graduate of London University, first carried out research at Battersea Polytechnic, London. From 1938 to 1949 he was a research and colour chemist at the Wool Industries Research Association, Leeds. In 1949 he was appointed to his present position of Research Biochemist at the Canadian Red Cross Memorial Hospital, Taplow, Berkshire, where he is chiefly engaged on investigations connected with juvenile rheumatism. He was one of the team which, in 1944, introduced the modern form of paper chromatography, followed by the complementary techniques of ionophoresis in silica jelly and chromatography on columns of ion-exchange resin. These methods were employed for determining amino-acid sequences in proteins and polypeptides, a notable example being gramicidin S (*Biochem. J.* 1947, **41**, 596). Another new technique with which he has been concerned, and which is being used to investigate protein-polysaccharide complexes, is the ionophoretic separation of sugars on paper (*Nature, Lond.* 1952, **169**, 783).

Dr C. E. Dent was, from 1935 to 1937, chemist in the Research Department in the Imperial Chemical Industries (Dyestuffs Group) Ltd., Manchester, and, since 1944, has been assistant in the Medical Unit at University College Hospital Medical School, London. His past work has been concerned with the chemistry of the phthalocyanines, and with the applications of biochemistry to clinical medicine. Dr Dent is at present Honorary Physician at University College Hospital, London, where he is in charge of the metabolic ward; Reader in Medicine in the University of London; Honorary Lecturer in Biochemistry, University College, London; and Honorary Advisor in Metabolic Studies to the Royal National Orthopaedic Hospital, London.

Dr J. E. Falk, after graduating B.Sc. in the University of Sydney, worked as a Wellcome Research Fellow, later being awarded the degree of M.Sc. He held a Fellowship of the Australian National Health and Medical Research Council, and in 1949 came to England under the auspices of the Nuffield Foundation to continue his work on problems of porphyrin chemistry and biochemistry under Professor C. Rimington, at University College Hospital Medical School, London. In 1951 he was admitted to the degree of Ph.D. in the University of London. In the same year a Nuffield Unit for the Investigation of Pyrrole Pigment Metabolism was established under Professor Rimington, with Dr Falk as leader of the Unit, a position he still holds. Dr Falk is an honorary lecturer in biochemistry in University College, London, and was appointed in 1953 to a Foulerton Research Fellowship by the Royal Society. The work of Dr Falk and the Nuffield Unit embraces many aspects of the wide field of porphyrin chemistry and biochemistry, and recent publications in *Nature* and in the *Biochemical Journal* have marked important advances in the field of porphyrin and haemoglobin biosynthesis.

Dr Jack Gross has previously contributed to the Bulletin (*Brit. med. Bull.* 1952, **8**, 136) an article on the experimental study of thyroid metabolism with radioactive iodine, with which a biographical note was published. Dr Gross is currently Associate Professor of Anatomy at the State University of New York Medical Center at New York City.

Dr F. A. Isherwood is a principal scientific officer at the Low Temperature Research Station, University of Cambridge and Department of Scientific and Industrial Research. His work has covered a wide range of topics connected with food, including the dehydration of vegetables. Since the Second World War, the investigation of the cell wall of plants and the development of new methods for the analysis of acids, amino acids, sugars (the results of this work were published in *Biochem. J.* 1951, **48**, 515), phosphoric esters and α-keto acids in biological extracts have been his major interests. More recently he has been concerned with investigations of the route by which vitamin C is synthesized in both the plant and the animal (*Biochem. J.* 1953, **56**, 1; 22). This work has elucidated not only the main features of the route but has shown that enzymes which will catalyse several of the intermediate reactions can be extracted from both plant and animal tissues.

Dr A. T. James has, since 1950, been a member of the Scientific Staff of the National Institute for Medical Research, London, where he is working on the development of gas–liquid chromatography with Dr A. J. P. Martin. From 1947 to 1950 he worked at the Lister Institute of Preventive Medicine, London—first as a member of the Scientific Staff, and later as Beit Memorial Fellow for Medical Research. At this Institute he worked with Dr R. L. M. Synge on the study of non-peptide linkages in gramicidin, and with Professor W. T. J. Morgan on the separation and estimation of the amino sugars present in mucopolysaccharides. He has published a number of papers (some of them with Dr A. J. P. Martin) on gas–liquid chromatography (*Biochem. J.* 1952, **50**, 679; **52**, 238; **52**, 242).

Dr Tudor S. G. Jones graduated in chemistry (B.Sc.) and physiology (Ph.D.) from the University of Wales. He has worked at the Cardiff City Mental Hospital, Runwell Mental Hospital, Wickford, Essex, the National Institute for Research in Dairying, Shinfield, Berkshire, and the University and Royal Infirmary, Manchester. He is now in charge of physical chemistry research at the Wellcome Research Laboratories, Beckenham, Kent. With the general aim of investigating the relationship between chemical structure and biological activity, he is mainly interested in the detailed structure of molecules, the general approach to this being through instrumental methods such as spectroscopy and electrochemistry. His published work includes papers on the biochemistry of milk secretion, antipernicious anaemia substances, epilepsy, chromatography, and the chemistry of antibiotics.

Professor J. W. H. Lugg at present occupies the Rockefeller Chair of Biochemistry in the University of Malaya. He was educated at the University of Western Australia. He was awarded the Rennie Memorial Medal in 1932 and the H. G. Smith Medal in 1944. From 1929 to 1945 he was a member of the staff of the Australian Council for Scientific and Industrial Research. From then until 1948 he was Senior Lecturer in Plant Biochemistry in the University of Melbourne. His previous work includes studies on the theory of solutions, plant proteins, and plant organic acids. His publications include "The chemistry and metabolism of the compounds of sulfur" (*Annu. Rev. Biochem.* 1945, **14**, 263), "Plant proteins" (*Advanc. Protein Chem.* 1949, **5**, 229), and a series of original papers on the same topics. He is at present extending his studies of plant proteins, and is engaged in work on ketosteroid excretion in different ethnic groups.

Dr Roy Markham was appointed to the staff of the Plant Virus Research Station of the University of Cambridge School of Agriculture in 1940. In 1946 this station was transferred to the direction of the Agricultural Research Council under the name of the Plant Virus Research Unit, which became attached to the Molteno Institute, Cambridge. During this time Dr Markham has been concerned with the study of the plant viruses, more particularly from a biochemical standpoint, and for the last few years he has been mainly engaged in the study of nucleic acids, especially those from viruses. He has published, jointly with other authors, papers on the

sizes of viruses and the methods employed in their estimation (*Parasitology*, 1942, **34**, 315), on the virus of turnip yellow mosaic (*Parasitology*, 1947, **39**, 330) and on the structure of ribonucleic acid (*Biochem. J.* 1952, **52**, 552).

DR A. J. P. MARTIN worked, from 1933 to 1938, at the Dunn Nutritional Laboratory, Cambridge, where he attempted the isolation of vitamin E, using chromatography and solvent extraction in the studies, and where he later worked under Sir Charles Martin on the B_2 group of vitamin deficiencies in pigs. From 1938 to 1946 he worked at the Wool Industries Research Association, Leeds, on the felting of wool and, in collaboration first with Dr R. L. M. Synge and later with Drs R. Consden and A. H. Gordon, on amino-acid analysis, using partition chromatography with silica gel and paper. In 1946 he became Head of the Biochemistry Division of the Research Department of Boots Pure Drug Company Ltd., Nottingham. He held this post until 1948, since when he has been on the Scientific Staff of the Medical Research Council: first at the Lister Institute for Preventive Medicine, London, and later at the National Institute for Medical Research, London, where he is now Head of the Division of Physical Chemistry. Dr Martin has, with Dr A. T. James, recently developed the method of gas–liquid chromatography. In 1950 he was elected a Fellow of the Royal Society. He was awarded, in 1951, the Berzelius Gold Medal of Svenska Läkaresällskapet and, in 1952, the Nobel Prize in Chemistry (jointly with Dr R. L. M. Synge) for work on partition chromatography. He is joint author of "A new form of chromatogram employing two liquid phases" (*Biochem. J.* 1941, **35**, 1358), "Qualitative analysis of proteins: a partition chromatographic method using paper" (*Biochem. J.* 1944, **38**, 224) and "Gas–liquid partition chromatography: a technique for the analysis of volatile materials" (*Analyst*, 1952, **77**, 915).

DR S. M. PARTRIDGE is a graduate of the University of London. After postgraduate research in organic chemistry under Dr J. Kenyon, he received his early biochemical training under Professor W. T. J. Morgan, at the Lister Institute of Preventive Medicine, London. He held a Beit Memorial Fellowship from 1939 to 1941 and afterwards joined the Department of Scientific and Industrial Research as a member of the staff of the Food Investigation Organization. He is now Senior Principal Scientific Officer at the Low Temperature Research Station, Cambridge, which is the central laboratory of the Organization. Dr Partridge's work since 1945 has concerned the study of the protein and mucopolysaccharide constituents of connective tissue, but during this period he has also been particularly interested in the development of chromatographic and other methods for the separation of the complex mixtures encountered in biochemical research. Among his contributions in this field were the first demonstrations of the application of paper chromatography to the identification of substances in the carbohydrate series, and the development of the use of ion-exchange chromatography for the isolation of amino acids and bases.

DR F. H. POLLARD is Reader in Inorganic Chemistry in the University of Bristol, where he has been a member of the staff since 1941. Previously he held teaching appointments in Swindon, Wiltshire, in Thornbury, Gloucestershire, and in Stoke-on-Trent, Staffordshire. In addition to his work on inorganic chromatography, he is interested in kinetics and combustion problems of chemical systems containing oxides of nitrogen. He has also published papers on the reactivity of inorganic nitrides, and on the behaviour of the chlorides of transition elements with ammonia. He is the joint author, with Dr J. F. W. McOmie, of a monograph, *Chromatographic methods of inorganic analysis* (1953). During the period 1948–54 he has been Local Representative of the Chemical Society in the Bristol area.

DR R. R. PORTER took his first degree at the University of Liverpool in 1939, and from 1939 to 1940 he was Johnston Colonial Fellow in Biochemistry there. From 1940 to 1946 he served in the Army, chiefly in the North African and Central Mediterranean theatres. From 1946 to 1948 he worked with Dr F. Sanger for his Ph.D. degree at the University of Cambridge where, after graduating, he continued to work under the auspices of the Medical Research Council. Since 1949 he has worked at the National Institute for Medical Research, London. Dr Porter's principal research interest has been in protein structure, particularly in the relation of biological activity to structure. He has published papers on a chemical study of rabbit antiovalbumin and the production of a specific inhibitor from it (*Biochem. J.* 1950, **46**, 473; 479), and on the partition chromatography of insulin and other proteins (*Biochem. J.* 1953, **53**, 320); he has also contributed a chapter, on the relation of chemical structure to the biological activity of proteins, to *The proteins*, vol. 1 (1953), edited by H. Neurath & K. Bailey.

DR J. M. WALSHE is at present Stothert Research Fellow of the Royal Society at University College Hospital Medical School, London. He has published the following papers: "Observations on the symptomatology and pathogenesis of hepatic coma" (*Quart. J. Med.* 1951, **20**, 421), "The effect of glutamic acid on the coma of hepatic failure" (*Lancet*, 1953, **2**, 1075) and "Disturbances of amino-acid metabolism following liver injury" (*Quart. J. Med.* 1953, **22**, 483).

DR R. J. P. WILLIAMS is a Research Fellow of Merton College, Oxford. He has been interested in the study of the stability of complex ions and its relevance to the function of metal ions in biological systems, and has published several papers on these topics. From 1950–51 he visited the University of Uppsala, Sweden, working with Professor A. Tiselius on the development of new methods in chromatography. This work is referred to in his contribution to this number of *British Medical Bulletin*.

DR T. S. WORK has been a member of the Scientific Staff of the Medical Research Council since 1938. Prior to that date he worked at the Lister Institute of Preventive Medicine, London, and was Lecturer in Biochemistry at the University of Edinburgh. His work and scientific publications have been concerned with the chemistry of natural products (1934–38), with the synthesis of chemotherapeutic drugs, particularly antimalarial drugs (1938–45), with antibiotic peptides (1946–50) and, since 1953, with the biosynthesis of proteins. Dr Work has also published, jointly with Mrs Work, *The basis of chemotherapy* (1948).

* * *

GENERAL PRINCIPLES OF CHROMATOGRAPHY

R. J. P. WILLIAMS M.A. D.Phil.

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1 Early experiments in adsorption chromatography
2 Partition chromatography and elution analysis
3 Frontal and displacement analysis
4 Carrier displacement
5 Gradient elution analysis
References

It would now appear that the use of chromatography as a simple separative technique has a history of more than one hundred years (Williams & Weil, 1953). It was not until the work of Kuhn, Winterstein & Lederer (1931), however, that the importance of the method was recognized. The latter workers employed the same technique as the botanist, Tswett (1910), whose book on chromophylls in the plant and animal world, which described chromatographic processes, had been ignored for thirty years.

1. Early Experiments in Adsorption Chromatography

A brief description of Tswett's method will illustrate its extreme simplicity. Tswett, and similarly Kuhn and co-workers, took a small volume of a petroleum ether extract of green leaves and poured it on to a column of powdered calcium carbonate, packed in a glass tube. He noted that the coloured material was retained at the top of the column as a small band. He then added pure petroleum ether to the top of the column, when the band of the coloured substance not only commenced to migrate but split up into several discrete zones of material of different colours. In this way chlorophyll was shown to be composed of a number of components —the different coloured bands. The separated components can be obtained from the column by breaking it up or preferably by continuing the washing with pure solvent until each zone has been washed, in turn, from the bottom of the column. Chlorophylls *a* and *b*, which can be separated in this way, differ from one another only by the replacement of a $—CH_3$ group by a —CHO group in a molecule of formula $C_{55}H_{72}N_4O_5Mg$. This represents one of the first, but only one, of many other remarkable separations achieved by the above simple procedure.

The separation method devised by Tswett is dependent upon the differences in the distributive behaviour of individual components between the two phases of the column—the solid calcium carbonate phase and the flowing liquid, petroleum ether, phase. If a very thin cross-section of the column is considered during the operation of an experiment, the fraction of a solute A, e.g., chlorophyll *a*, in the liquid phase can be written α and the fraction in the solid phase will then be $(1 - \alpha)$. For a solute B, e.g., chlorophyll *b*, the stationary fraction on the solid phase can be put equal to $(1 - \beta)$, when the fraction in the mobile liquid phase which runs through the column will be β. The distribution ratio $\alpha/(1 - \alpha)$ will generally differ from $\beta/(1 - \beta)$ and so the possibility of partially separating A from B occurs by separating the liquid and the solid phases in the column, i.e. by flowing the liquid. This separation could have been carried out in bulk in a conical flask by shaking a solution of A and B in petroleum ether with calcium carbonate and then decanting off the liquid. If β approximates to unity and α to zero, then complete separation of A from B could be managed in this single operation, as substance A would be totally retained by the calcium carbonate whereas B would be found in the decanted liquid. This simple technique is the method used by the organic chemist in the purification of compounds from contamination with coloured materials, usually of higher molecular weight, when he shakes a solution of the material with powdered charcoal. It is unusual to find such large differences between α and β, however, and therefore it is often necessary to repeat the above operation by shaking the filtrate with more adsorbent and the residual adsorbent with more solvent. Such an extension of a single distribution to a many-stage fractional extraction has been repeatedly illustrated, by reference, for example, to the separation of rare earth metals, and it will not be further described here. Chromatography is a means of bringing about this many-stage separation, each stage taking only a small length of the adsorbent column. It is important to realize, however, that in fractional extraction any unequal distribution of the different components between two immiscible phases brings about partial separation. The nature of the two phases is a matter of convenience and apart from distribution between a liquid and a solid surface, as used by Tswett, there are the possibilities of using distribution between two immiscible liquid phases, a gas and a liquid phase, or a gas and a solid phase. Such processes as distillation and partition are very similar to adsorption on to a solid from a liquid, each involving a change of phase of a part of the material.

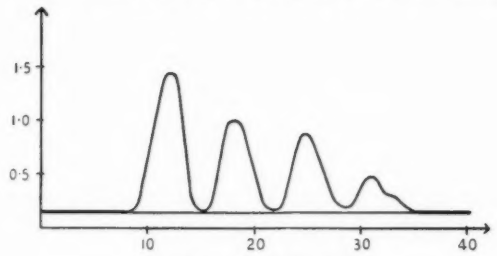
2. Partition Chromatography and Elution Analysis

While it has long been realized that a many-stage fractional distribution could be used to separate components of a mixture which differed but slightly, i.e., for which $\alpha/(1 - \alpha)$ and $\beta/(1 - \beta)$ were nearly equal, the processes involved were extremely tedious. For this reason, shortly before the nature of chromatography was realized, Martin and Synge in Great Britain and Craig & Craig (1950) in the USA devised machines which could automatically perform the operations involved in partition experiments. The machines are comprised of a large number of separating funnels each of which contains a definite volume of the two immiscible liquid phases between which partition will occur. The funnels are automatically agitated for a given time at the end of which the lighter of the liquid phases in each funnel is automatically transferred to the next funnel. The cycle of operations—shaking for a given period followed by transfer of the lighter phase—is then repeated. Consideration of such a machine shows it to be a counter-current liquid-liquid extractor in which each funnel is equivalent to a single stage in a normal many-stage fractional extraction. An experiment with the machine is commenced by placing the material to be examined in one of the funnels. Whereas Craig has developed this machine, Martin and Synge realized that the multi-stage process could be achieved in

another way on a smaller scale but with no complex apparatus. Their method resembles closely that of Tswett in so far as experimental detail is concerned. In place of Tswett's solid phase, calcium carbonate, Martin and Synge used silica gel, and for the flowing phase they found that chloroform was suitable in the examination of the acetylated amino acids. Following the method of Tswett, the latter substances were added to the top of the column of silica gel in a small volume of solvent and then the band of materials formed was washed and developed, with additional pure solvent. Mixtures of many acetylated amino acids were resolved into single component bands travelling down the silica gel column at different rates. The close parallel between the method of Tswett and that of Martin and Synge hides a difference in principle. In Tswett's experiments separation was brought about by differences in distribution between a solid surface and a liquid solution—adsorption chromatography. In Martin and Synge's experiments, however, the separation is due to differences in distribution between two liquid phases—partition chromatography: the mobile chloroform phase being one and the other being the stationary liquid phase, water, which is held in the pores of the silica gel. However, as far as practical considerations are concerned the underlying mechanism of chromatography—be it adsorption or partition—is often unimportant, both processes bringing about the same result. In discussing their experiments Martin & Synge (1941) considered their column to consist of a large number of theoretical stages, each stage equivalent to one separating funnel in a many-stage fractional partition, and they were able to show mathematically that the experimental separations on the column followed a pattern predictable from such a simple initial assumption and from a knowledge of the values of the different distribution coefficients, i.e. $\alpha/(1-\alpha)$ and $\beta/(1-\beta)$. Thus chromatography was shown to be a multi-stage countercurrent, or column, process. The simple one-stage separation methods, such as partition in a separating funnel, are related to it in a fashion analogous to the relationship between simple distillation and fractional distillation in a column.

The separation of zones of different compounds in a chromatogram is restricted by a consideration omitted in the above discussion. It has been assumed so far that the ratio $\alpha/(1-\alpha)$ has a fixed value for one substance. However, it is known that this value often varies for a given compound as its concentration varies, i.e. Nernst's law does not hold. Now, in a chromatographic column, diffusion of material occurs and the concentration of a given compound will change along the length of the column so that instead of a substance forming a zone of constant concentration, as was initially obtained at the top of the column on introducing the material, as the experiment continues the zone will approximate more and more closely to a Gaussian error curve with a wave-like form. If the value of $\alpha/(1-\alpha)$ does not vary with concentration then this wave (elution peak as it is called) travels through the column as a symmetrical zone and diffusion does not greatly influence separation (see fig. 1). However, if $\alpha/(1-\alpha)$ varies with concentration, the front and tail of the zone travel down the column at a different rate from the maximum concentration, for, as pointed out above, it is this ratio which controls the migration rate. Two types of unsymmetrical zones can be obtained in this way according to the way in which $\alpha/(1-\alpha)$ varies with concentration, c. If it increases with concentration, the fraction in the mobile phase increases with concentration, and the elution peak has a sharp leading edge and a

FIG. 1. A PLOT OF THE CONCENTRATION OF AMINO ACIDS, AS MEASURED BY A COLORIMETRIC DETERMINATION, AS THEY ARE ELUTED IN SYMMETRICALLY SHAPED ZONES IN DIFFERENT VOLUME FRACTIONS OF THE EFFLUENT SOLVENT FROM A CHROMATOGRAPHIC COLUMN



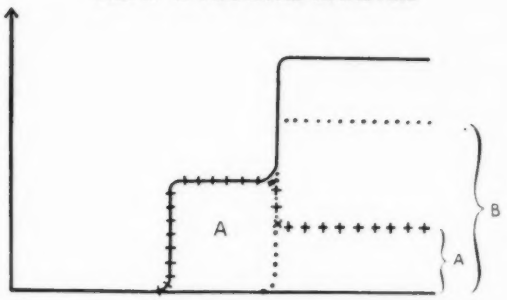
long tail. At the low concentrations in the tail, $\alpha/(1-\alpha)$ of substance A may be smaller than $\beta/(1-\beta)$ of substance B at high concentration of B, so that the tail of A and the maximum concentration of B overlap and separation cannot be achieved by elution. Likewise if $\alpha/(1-\alpha)$ decreases with concentration, owing to co-operative phenomena, separation is again made more difficult by the spreading of the zone, due to the different rates of movement of its different concentration regions.

3. Frontal and Displacement Analysis

Tiselius has devised two alternative methods of chromatographic analysis: frontal analysis and displacement analysis (Tiselius, 1943; cf. Claesson, 1946). Neither of them is applicable to substances for which $\alpha/(1-\alpha)$ is a constant independent of concentration, that is for substances obeying linear adsorption or partition isotherms, but both apply to substances having a distribution which varies with concentration in such a manner that the ratio $\alpha/(1-\alpha)$ increases with increase in concentration. The methods are therefore complementary to elution analysis. In such cases, although this is primarily true of adsorption, it is frequently found that the distribution of one substance is profoundly affected by the distribution of another. Substances can be said to "displace" one another from the surface of an adsorbent.

Consider then the result of running a large volume of a solution of two components, which have curved adsorption isotherms ($\alpha/(1-\alpha)$ increases with c) down a column of adsorbent. The zones formed in such an experiment can be readily seen to have sharp fronts rising immediately to the maximum concentration in the zone (see fig. 2), for if material diffuses out in front of this zone it will be in a region of low concentration where $\alpha/(1-\alpha)$ will be smaller—and therefore where the rate of migration will be slower—than in the region of higher concentration immediately behind it. The region of lower concentration will therefore be overtaken by the higher concentration and a square front will obtain. The speed of travel of the fronts of different substances will not be the same, however, so that there will be two sharp fronts travelling down the column, corresponding to the two substances. Although this method, frontal analysis, can be used analytically it cannot be used to estimate the amounts of the materials in the mixture.

FIG. 2. A FRONTAL ANALYSIS



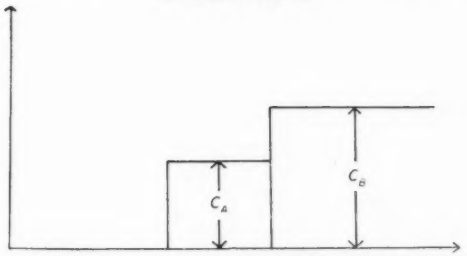
Abscissae: effluent volume
Ordinates: solute concentration

The concentration of two materials, A and B, and the total concentration are plotted against the corresponding volume reading as the solvent leaves the chromatographic column.

Concentration of A ++++++++++
Concentration of B .....
Total concentration ——————

If, after a small volume of the above solution of two components had been put on to the column, a solution of a third component in the same solvent had been added, and if this latter component was the most strongly adsorbed, then a displacement of the two initial components would have taken place. Such displacements lead to zones of pure substances moving down the column in the order of their adsorption strengths (fig. 3). The height of a step in the displacement diagram, that is, the concentration at which a given substance travels down the column, is a specific property of a given substance under fixed conditions of displacement. Furthermore, under fixed conditions the step length is proportional to the amount of the substance. Displacement analysis is a quantitative analytical method and it has the advantage that it can be used on a preparative scale. Figure 3 represents a steady state at equilibrium, and, not as in elution analysis, displacement is not improved by increasing column length beyond a certain value at which this equilibrium state

FIG. 3. A DISPLACEMENT OF SUBSTANCE A BY SUBSTANCE B



Hagdahl, Williams & Tiselius (1952) by permission of Arkiv för Kemi

Abscissae: effluent volume
Ordinates: solute concentration

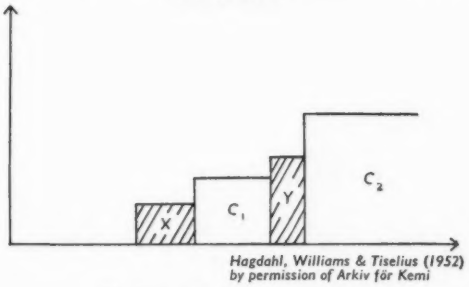
The concentration of A (C_A) and of B (C_B) are plotted against the corresponding volume reading as the solvent leaves the chromatographic column.

is achieved. It must, however, be added that both frontal analysis and displacement are not always so simple as described here and many complications have been observed. Neither is the differentiation between elution and displacement so readily made as in the above illustrative examples. For further discussion reference is made to recent papers by Hagdahl, Williams & Tiselius (1952) and Williams, Hagdahl & Tiselius (1954), where such matters are discussed at length.

4. Carrier Displacement

In displacement analysis components travel down the column adjacent to one another and thus, although the method has some advantages over elution, complete separation of materials is not possible. In order to overcome this difficulty Tiselius suggested the method of carrier displacement analysis in which substances C_1 and C_2 are deliberately added to those, x and y, which are to be separated. C_1 and C_2 are so chosen that C_1 displaces x, but not y, and C_2 displaces x and y and C_1. The displacement diagram should then consist of the steps of x, C_1, y and C_2 in this order and x and y travel well separated from one another down the column (fig. 4). The

FIG. 4. A CARRIER DISPLACEMENT DIAGRAM, IDEALIZED



Hagdahl, Williams & Tiselius (1952) by permission of Arkiv för Kemi

Abscissae: effluent volume
Ordinates: solute concentration

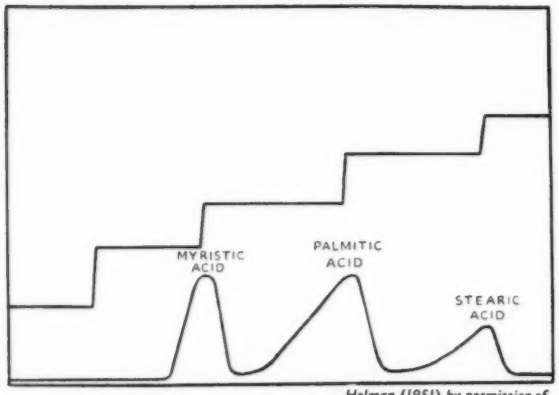
The manner of plotting this figure is the same as in fig. 3.
x, y: substances to be separated
C_1, C_2: substances added to x and y

method does not appear to be as successful as had been hoped, for it is necessary to choose C_1 and C_2 so that they can be separated from x and y, chemically, after the chromatogram has been run. This condition is unfortunate in so far that it is now recognized that displacement does not occur readily between substances which are chemically very different (Hagdahl *et al.* 1952). Figure 5 represents the type of displacement diagram which is to be expected in such carrier experiments. It can be seen to resemble an elution in the zones of the carriers.

5. Gradient Elution Analysis

In order to overcome some of the difficulties with displacement a further chromatographic method has been introduced —gradient elution analysis (Alm, Williams & Tiselius, 1952). It combines the techniques of displacement with those of elution and it will be discussed at some length here in order to indicate its advantages and limitations. The method has not

FIG. 5. AN ACTUAL CARRIER DISPLACEMENT, SHOWING FATTY ACIDS ELUTED IN THE ZONES OF ESTERS OF THE SAME FATTY ACIDS THAT ACT AS CARRIERS



Holman (1951) by permission of Journal of the American Chemical Society

Abscissae: effluent fractions

Ordinates:

Left-hand: refractive index of displacer steps

Right-hand: alkali titre of acids per fraction

The steps are the carrier zones, and the peaks are the zones of the acids, plotted as concentration against the corresponding effluent volume.

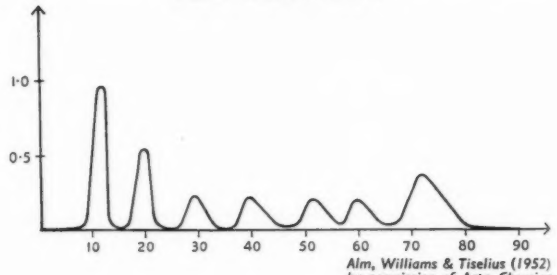
been widely used as yet, except inadvertently (see later). It consists in the elution of material from an adsorbent column by a continuously modified solvent, commencing the elution with a solvent which will not elute the components but in which the conditions for displacement apply (curved isotherms), and then continuously changing the initial solvent, using an external mixing vessel, by adding a second solvent in which the components of the mixture are not adsorbed at all. The components then travel as elution peaks (linear isotherms) towards the end of their passage through the column. Thus, although displacement brings about much of the separation achieved, the zones of the components are finally separated from one another. The first successful experiments carried out using this method are illustrated in fig. 6 (Alm *et al.* 1952). Later experiments described the separation of both saccharides and of amino acids and peptides (Alm, 1952; Williams, 1952).

An interesting extension of the method has been made to the fractionation of high polymers (Bannister, Phillips & Williams, in preparation). Previously no fractionation of high polymers had been successfully made by chromatographic methods, although Claesson (1949) had analysed polymers by a rather uncertain mathematical examination based upon frontal analysis. With gradient elution the fractionation of silicone polymers has proved to be particularly satisfactory. These polymers are liquids and can be applied to carbon columns directly. They are not eluted by methanol but ethyl ether elutes them quantitatively. Thus by use of a gradually increasing concentration, a gradient of ether in methanol, the polymers can be eluted fraction by fraction. The highest polymers come from the adsorbent in the best solvent, so long as members of only one homologous series are present, but if there is a mixture of polymer types then the separation achieved is not simply related to molecular weight (see fig. 7). (Silicones can be obtained which are straight-chain dimethyl silanes but others are available which contain large numbers of phenyl residues.) The highest polymer examined as yet was one of average molecular weight—100,000.

Gradient elution methods are also applicable to other adsorbents, and attention has been directed to work with carbon only because of the detailed knowledge of adsorption on to this material. However, Donaldson, Tulane & Marshall (1952) in the USA have used gradient elution analysis in the examination of complex mixtures of substituted fatty acids. Their adsorbent was silica gel and they employed an increasing gradient of amyl alcohol or butanol in chloroform. In the separation of enzymes Mitchell, Gordon & Haskins (1949) have applied a gradient of salt concentration to the elution of enzymes, using a filter-paper chromatopile as an adsorbent. Both these groups of authors reported considerable success with the method.

Very recently gradient elution analysis has been applied to inorganic chromatography with immediate success. Lederer (1953) has applied it in an improved separation of a number of cations on paper strips. Chalkley (1953) has shown how to extend the method to column procedures, using gradients of acid concentration in the analysis of mixtures of Zn, Hg, Cd, Fe, Co, Ni and Cu. A point of great interest which has been established by his work is that many of the separations of inorganic ions previously carried out by elution from cellulose columns automatically used gradients of solvents. The gradients were established in the column, and not external to it, by the experimental method employed in setting up the columns. For example, if a three-component system of 3 N-HCl–water–ethyl ether is equilibrated and the organic layer used to wash a cellulose column packed in water, then it is found that the acid concentration in the effluent does not reach an equilibrium value over a considerable volume of solvent. In cases where the column is packed in the heavier aqueous phase of the above three-component system, a gradient of acid and water content is again obtained over the length of the column. There can be little doubt that the gradients fortuitously obtained have aided many separations and have obscured the nature of the chromatography of cations on cellulose columns (cf. Wells, 1953).

FIG. 6. GRADIENT ELUTION ANALYSIS OF SEVEN POLYSACCHARIDES



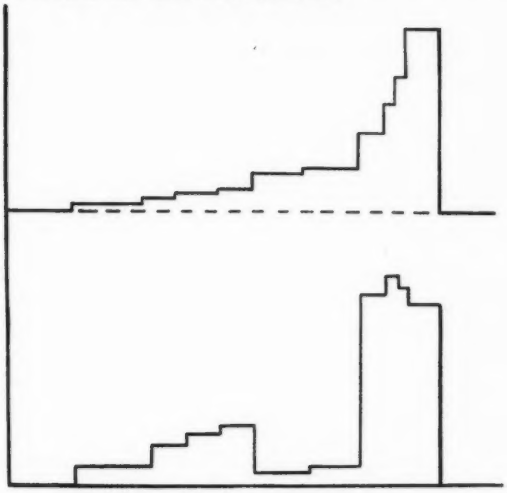
Alm, Williams & Tiselius (1952) by permission of Acta Chemica Scandinavia

Abscissae: fraction number

Ordinates: optical density

The method of plotting is the same as in fig. 1.

FIG. 7. ANALYSIS OF A SILICONE POLYMER, USING A GRADIENT ELUTION METHOD (after Bannister, Phillips & Williams, in preparation)



Abscissae: volume of effluent

Ordinates:

Upper: viscosity of polymer fractions

Lower: concentration of polymer

The viscosities were measured at a fixed concentration.

The deliberate use of a gradient of pH along a column has been found to be useful in the separation of alkaloids on a partition column (Carless, 1953). In this case the stationary phase was initially buffered at pH 3.0 and a dilute solution of pyridine was employed as the eluting solution. As the pyridine is taken into the stationary phase it modifies the pH of the latter. The final effluent pH in the experiments of Carless was 4.5. The gradient in this case depends upon a number of variables such as the buffer capacity. Carless showed that a greatly improved separation of ergocristinine and ergocristine was achieved through the gradient. There must be many other examples in the literature of chromatography where such pH gradients have been accidentally formed. It must not be forgotten, for example, that if an organic acid buffer is used as the stationary phase in a chromatogram using benzene, for example, as the mobile phase, then as the benzene will extract the organic acid, but not the acid anion, the pH of the column will rise steadily throughout the experiment. In such partition systems little real advantage accrues from the use of a gradient except in the reduction of the quantity of solvent used.

Finally, gradient elution has been frequently employed, accidentally, in chromatography on ion-exchange resins. In any elution in which step-wise changes of pH are attempted using ion-exchange resins as the adsorbent, e.g., in the analysis of amino acids, the buffering capacity of the column is such as to produce a gradual (gradient) pH change. This fortuitous arrangement has obvious advantages over the attempted step-wise alteration of pH.

The above examples illustrate the use of gradient elution methods but it must not be thought that these methods supersede elution and displacement analysis to any degree. The new technique is only to be advocated under conditions where the others fail (see Williams, 1952), and it is described in detail here at the expense of details of the earlier methods because many illustrative examples of the latter are given elsewhere in this number of the Bulletin.

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GAS–LIQUID CHROMATOGRAPHY

A Technique for the Analysis and Identification of Volatile Materials

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In the techniques described in other papers in this number of the Bulletin the substances required to be separated were distributed either between two equilibrated liquid phases or between a liquid and a solid or semi-solid, but chromatographic separations of volatile materials are equally possible in systems consisting of a gas and a solid or a gas and a liquid. The adsorption chromatography of gases (gas–solid system) has been studied a great deal (Claesson, 1946) but gas–liquid chromatography was never attempted until James & Martin (1952b) published its use in the separation of volatile fatty acids. As the mobile phase of the chromatogram is a gas it is possible to use long thin columns and still to obtain high rates of flow in distinction from liquid–liquid chromatography. In all chromatograms the main factor limiting efficiency is the rate of diffusion of the substances to be separated within the two phases; the gaseous nature of the mobile phase of gas–liquid chromatograms makes diffusion very rapid. The main factor then limiting efficiency is the rate of diffusion in the stationary liquid phase, and as the columns are generally run at temperatures between 65° C. and 200° C. the elevated temperature results in a high rate of diffusion owing to the higher molecular velocities. It can therefore be seen that high column efficiencies are to be expected even with high rates of flow of the mobile phase. Indeed analyses can be carried out more rapidly with gas–liquid than with liquid–liquid partition chromatograms. An additional advantage conferred by this technique lies in the fact that in general it is easier to detect small quantities of vapour in a gas than it is to detect small quantities of a solute dissolved in a solvent.

The arrangement used experimentally is such that the stationary liquid phase (a substance of high boiling-point and good thermal stability) is distributed over the surface of an inert solid (usually kieselguhr, diatomaceous earth) which is packed into the column maintained at the appropriate temperature by a vapour jacket. The substances to be separated are applied to the top of the packing and are then blown down the column by a stream of permanent gas, usually nitrogen. The substances separate according to their relative volatility and are detected by a suitable device as they leave the column in vapour form in the stream of permanent gas.

Of the many theoretically possible methods of detecting vapours in gases three have so far been used with success. As in our initial work on this problem we were interested in studying the separation of volatile acids and bases, the method of detection and estimation chosen was titration. In fig. 1 is shown a schematic diagram of the apparatus used. The gas on leaving the column passes into a titration cell containing a suitable solvent, usually water and a pH-sensitive indicator (usually phenol red). As soon as an acid emerges from the column it is trapped in the water in the cell and the resultant change in pH causes the indicator colour to change from red to yellow. A green-sensitive photoelectric cell situated behind the titration cell responds to the change in colour by a change in photo current; this is passed to a simple amplifier and causes the drive to an automatic burette to start up and so deliver dilute alkali to the titration cell until the indicator colour returns to red again, when the burette is switched off. A pen attached to the moving plunger of the automatic burette records its movement on a chart held on a drum rotating once per hour. In this way a graph is obtained showing a series of steps; each step denotes the appearance of an acid and the straight line between steps shows no acid to be emerging from the column. The whole process is automatic after the column has been loaded, the nitrogen has been turned on and the titration cell has been filled with the indicator solution. The amount of each acid in microgram equivalents is read directly from the graph. In fig. 2 is shown the separation of acetic, propionic, *iso*butyric, *n*-butyric acids and all the isomers of valeric acid, an 11 ft.[1] column being used, of which the stationary phase consists of a 10% solution of stearic acid in a silicone oil (DC550). The upper curve is obtained by graphical differentiation of the experimental curve and shows

[1] 1 ft. = 30.5 cm.—Ed.

FIG. 1. SCHEMATIC LAY-OUT OF APPARATUS FOR SEPARATING VOLATILE ACIDS OR BASES, USING TITRATION AS THE METHOD OF DETECTION AND ESTIMATION

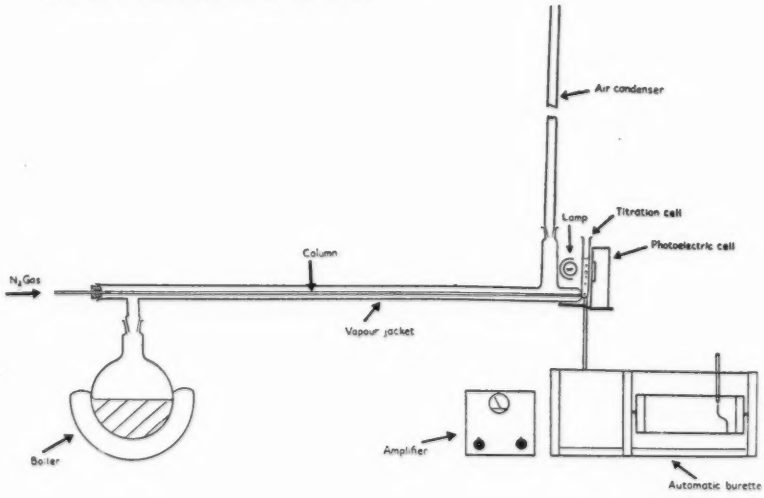
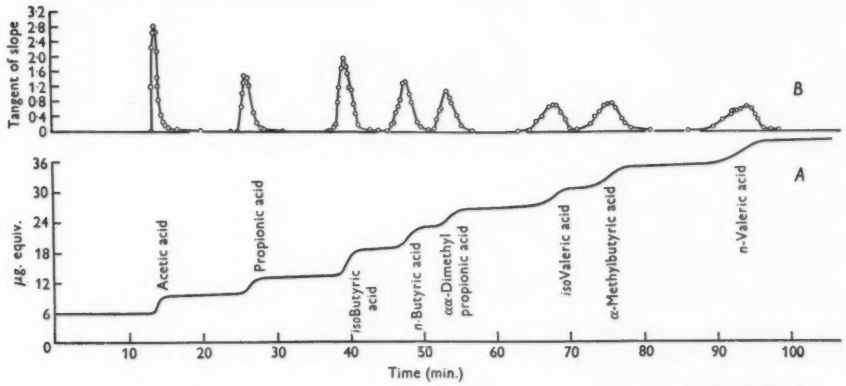


FIG. 2. THE SEPARATION OF ACETIC, PROPIONIC, *n*-BUTYRIC AND *iso*BUTYRIC ACIDS AND THE ISOMERS OF VALERIC ACID, SHOWING THE COMPLETE RESOLUTION OF ALL BANDS



James & Martin (1952b) by permission of Biochemical Journal

Curve A: experimental results

Curve B: differential of experimental curve

Column length, 11 ft.; stationary liquid phase, DC550 silicone fluid containing 10 % (w/w) stearic acid; temperature, 137° C.; rate of flow of nitrogen, 18.2 ml./min.; pressure of nitrogen, 74 cm. Hg

the peaks more usually encountered in chromatography. The whole analysis of the eight acids is complete in 100 minutes and the sensitivity is such that 0.02 mg. of acetic acid is detectable. The range of acids covered by this technique is from HCOOH to $C_{11}H_{23}COOH$, i.e., from formic to lauric acid. This technique has been used with success in studying the mode of biosynthesis of the fatty acids in liver and in the glycerides of milk fat (Popják & James, unpublished results, 1954), radioactive acetic acid being used as the precursor of the acids, and also in studying the volatile acids produced by various micro-organisms (Crowther, Fulton & Joyner, 1954).

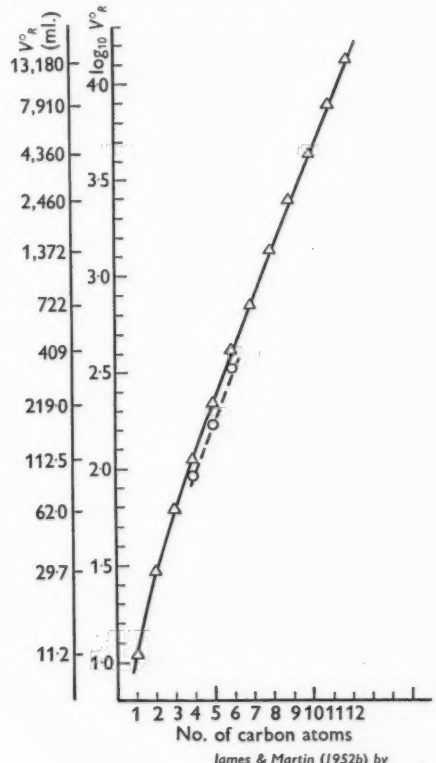
The time at which a given acid emerges from the column is constant at a constant temperature and flow-rate of nitrogen so that the time of emergence can be used in identifying an unknown acid. In a homologous series of fatty acids the energy of interaction with the stationary phase of the column increases by a constant amount for each increase of CH_2 in the chain length so that, if the logarithm of the time of emergence of each acid (or the volume of nitrogen emerging from the column before appearance of the acid, known as the retention volume V_R) is plotted against the number of carbon atoms for a series of acids, a straight line results (fig. 3). In this way, from knowledge of the behaviour of a few acids in the series, the behaviour of all the other homologues can be derived.

The columns can be used repeatedly without any obvious signs of deterioration. Where only qualitative results are required a higher flow-rate can be used and in fact all *n*- and *iso*-acids between formic acid and valeric acid can be identified in less than 20 minutes (James & Martin, 1952b).

The titration technique can be equally easily applied to volatile bases, and fig. 4 shows a separation of ammonia, mono-, di-, and tri-methylamines completed in 35 minutes. The rates at which different substances move down the column are dependent on the nature of the forces involved in their solution in the stationary phase. By variation of the nature of the stationary phase the order of appearance of the three methylamines can be altered in any way desired. The example shown uses a stationary phase (a mixture of a long-chain alcohol and a long-chain paraffin) such that the best separation of the four substances is obtained. By the use of a column with a stationary phase of glycerol the normal order of the three methylamines is reversed and a complete separation of the three may be obtained in eight minutes (James, Martin & Howard-Smith, 1952).

The separation of a wide range of aliphatic amines is shown in fig. 5: 15 amines are separated on a 4 ft. column whose stationary phase is liquid paraffin. The order

FIG. 3. THE RELATIONSHIP BETWEEN THE RETENTION VOLUME AT ZERO PRESSURE DIFFERENCE, V^0_R, AND THE NUMBER OF CARBON ATOMS IN THE LOWER FATTY ACIDS

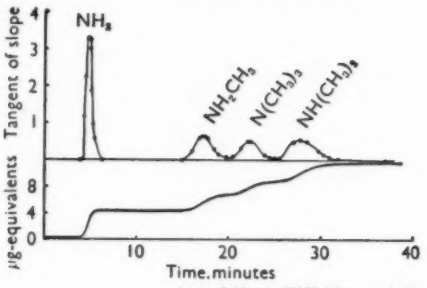


James & Martin (1952b) by permission of Biochemical Journal

△: *n*-acids ○: *iso*-acids

Data are for a 4-ft. silicone–stearic acid column at 137° C.

FIG. 4. THE SEPARATION OF AMMONIA AND MONO-, DI-, AND TRI-METHYLAMINES



James & Martin (1952a) by permission of the Society for Analytical Chemistry

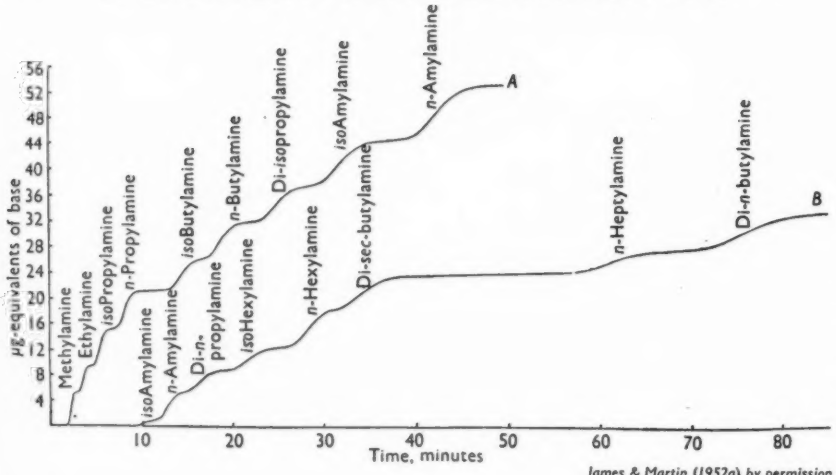
Lower curve: experimental results

Upper curve: differential of experimental curve

Column length, 4 ft.; stationary phase, hendecanol containing 15% (v/v) liquid paraffin; temperature, 78.6° C.; rate of flow of nitrogen, 5 ml./min.; pressure of nitrogen, 6.5 cm. Hg

in which the amines appear on this type of column is determined only by the molecular weight and degree of branching of the aliphatic chains, as the only solution forces are the so-called van der Waals or London "dispersion" forces. Stationary phases such as long-chain alcohols, ethers, or aromatic hydrocarbons alter the relative positions of the amines, because additional forces such as hydrogen bonding

FIG. 5. THE SEPARATION OF 15 ALIPHATIC AMINES ON A 4 FT. COLUMN, WITH LIQUID PARAFFIN AS THE STATIONARY PHASE, AT 100° C.



James & Martin (1952a) by permission of the Society for Analytical Chemistry

Curve A

Rate of flow of nitrogen, 5.7 ml./min.; pressure of nitrogen, 7.5 cm. Hg

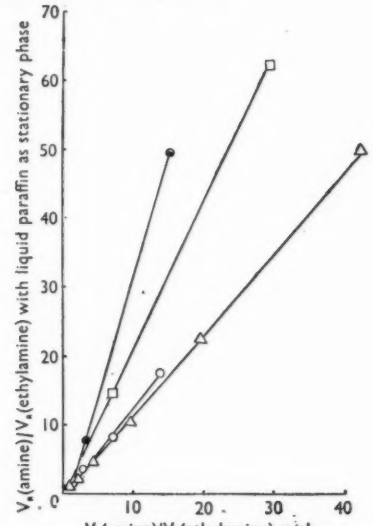
Amines in order of appearance: methylamine, ethylamine, *iso*propylamine, *n*-propylamine, *iso*butylamine, *n*-butylamine, di*iso*propylamine, *iso*amylamine (overlapping with triethylamine), *n*-amylamine

Curve B

Rate of flow of nitrogen, 18.7 ml./min.; pressure of nitrogen, 22.5 cm. Hg

Amines in order of appearance: *iso*amylamine, *n*-amylamine, di-*n*-propylamine, *iso*hexylamine, *n*-hexylamine, di-sec.-butylamine (overlapping with di*iso*butylamine and *cyclo*hexylamine), *n*-heptylamine (overlapping with tri-*n*-propylamine), di-*n*-butylamine

FIG. 6. THE RELATIONSHIP BETWEEN RETENTION VOLUME OF AMINE / RETENTION VOLUME OF ETHYLAMINE FOR COLUMNS HAVING STATIONARY LIQUID PHASES OF (i) LIQUID PARAFFIN AND (ii) LUBROL MO (A POLYETHYLENE OXIDE), SHOWING THE RELATIVE SPEEDING-UP OF SECONDARY AND TERTIARY AMINES ON CHANGING FROM A NON-HYDROGEN-BONDING SOLVENT TO ONE ALLOWING HYDROGEN BONDING


Vₐ(amine)/Vₐ(ethylamine) with liquid paraffin as stationary phase
Vₐ(amine)/Vₐ(ethylamine) with "Lubrol MO", as stationary phase

James & Martin (1952a) by permission of the Society for Analytical Chemistry

△ primary straight-chain amines

○ primary amines with an *iso*-configuration

□ secondary straight-chain amines

● tertiary straight-chain amines

are involved. A convenient stationary phase is a polyethylene oxide (Lubrol MO). In the aliphatic amines only primary and secondary amines are capable of hydrogen bonding with this as only these contain the necessary —N—H groups. Tertiary amines are for this reason unable to show hydrogen bonding. If the relative positions of the three types of amines on a liquid paraffin column (where only van der Waals forces operate) are plotted against their relative positions on such a poly ether column (where hydrogen bonding can occur), then it is found that the three types of amines fall on straight lines of different slope (see fig. 6). In this way the nature of an unknown amine may be closely defined (James, 1952).

Similar separations can be obtained with aromatic amines, as shown in fig. 7. In this case the amines are too feebly basic

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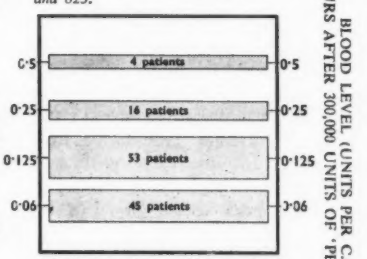

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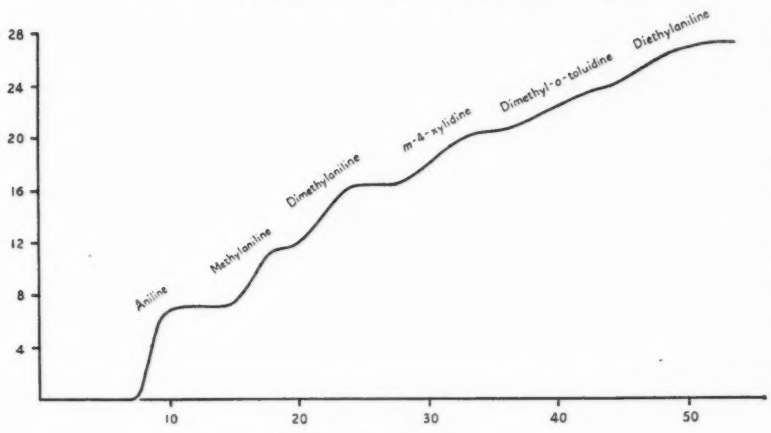
FIG. 7. THE SEPARATION OF AROMATIC BASES



Abscissae: time (min.)
Ordinates: μg. equiv. of base

Column length, 4 ft.; stationary phase, liquid paraffin; temperature, 137° C.; rate of flow of nitrogen, 75 ml./min.; pressure of nitrogen, 47.2 cm. Hg; titration carried out in glacial acetic acid with perchloric acid

Bases in order of appearance: aniline, methylaniline, dimethylaniline, *m*-4-xylidine, dimethyl-o-toluidine, diethylaniline

to be titrated in water so the titration was carried out in glacial acetic acid with perchloric acid, methyl violet being used as indicator (James, unpublished results, 1954).

In order to make the technique of gas–liquid chromatography applicable to all substances capable of being distilled, a more general method of detection of vapours in gases is necessary. Two methods have been developed and were originally used by Claesson (1946) in his study of gas adsorption chromatography.

The first method utilizes the fact that the thermal conductivity of a gas is changed by the presence of small amounts of foreign substances. The instrument (the catharometer) usually consists of four channels containing fine platinum wires heated electrically and arranged to lie along the axis of the channels. When a gas passes down a channel the wire is cooled to an extent dependent on the thermal conductivity of the gas, and the resistance of the wire changes. By using two comparator channels along which only the pure gas passes and by connecting the two running channels and the two comparator channels in a bridge circuit, it is possible to detect very low concentrations of material in a gas stream. This type of instrument has been used with great success by Ray (1954) in studying the analysis of hydrocarbons, esters, ethers, etc., with gas–liquid columns.

The second method takes advantage of the change in density of a gas stream caused by the presence of small amounts of volatile material. The instrument that we have developed measures the difference between the density of the gaseous effluent from the chromatogram and that from a comparison column through which only nitrogen is passed, the two columns being maintained at the same temperature and with the same nitrogen pressure. The apparatus consists essentially of a series of tubes bored in a copper block (held at the same temperature as the columns) and connected in a manner analogous to a Wheatstone bridge so that a difference of flow-rate of the two gas streams led into the block causes no pressure difference between two points in the system. Two sets of channels are connected by a cross-channel in such a way that any density difference between the two streams causes a pressure difference and hence a flow of gas through the cross-channel. This channel contains a flow detector consisting of a small filament (electrically heated) arranged below and equidistant from two connected thermojunctions; any cross-flow of gas causes the stream of hot convected gas from the filament to be diverted to one or other of the thermojunctions, heating one and cooling the other. The thermoelectromotive force produced is fed to a commercial d.c. amplifier whose output is led to a recording galvanometer. The galvanometer deflection is linearly related to the density difference of the two gas streams and, unlike the catharometer, is unaffected by changes in gas-flow rate. The instrument is highly sensitive, amounts

FIG. 8. SCHEMATIC LAY-OUT OF GAS–LIQUID PARTITION CHROMATOGRAPHY APPARATUS, USING A GAS-DENSITY BALANCE FOR DETECTION AND ESTIMATION OF THE ZONES

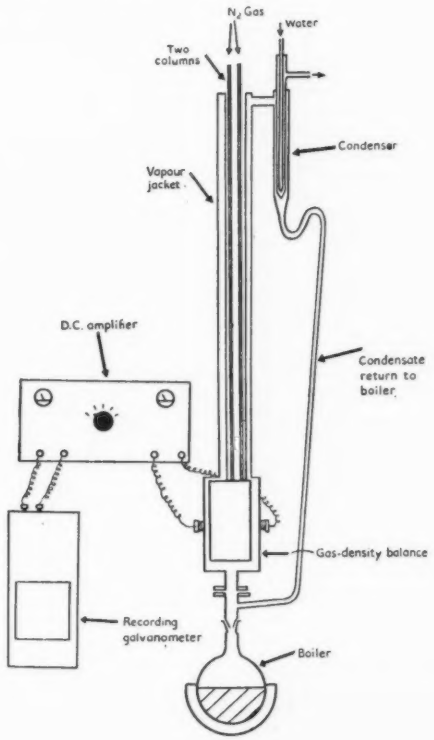
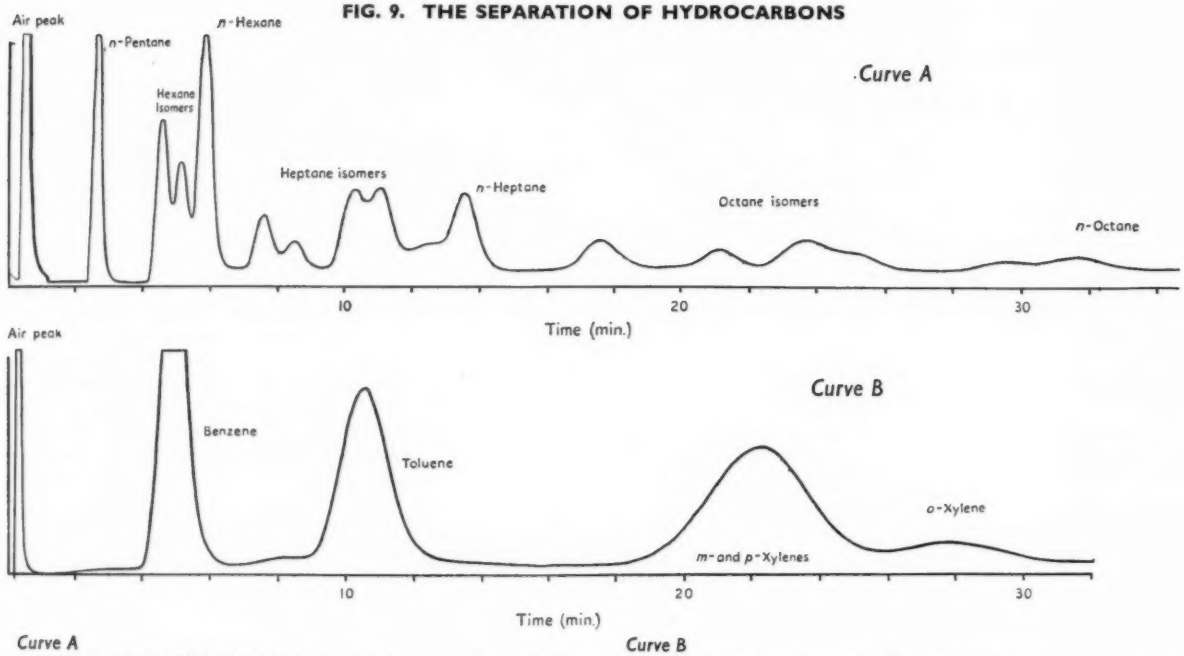


FIG. 9. THE SEPARATION OF HYDROCARBONS



Curve A

The separation of aliphatic hydrocarbons from petroleum ethers of boiling range 40°-120° C.

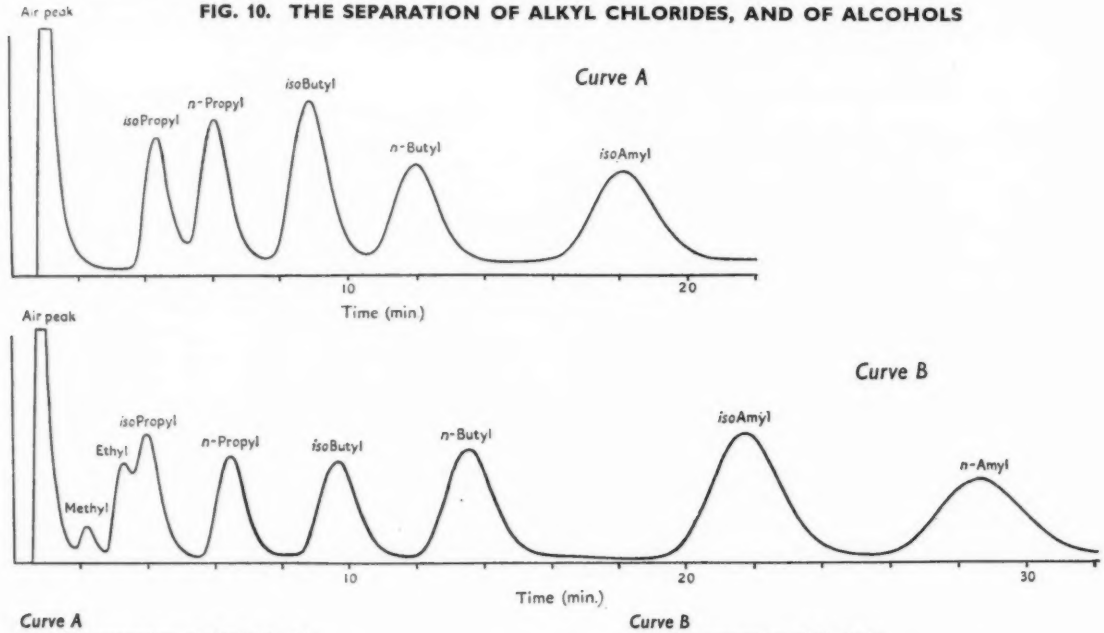
Column length, 4 ft.; stationary phase, liquid paraffin; temperature, 78.6° C.; rate of flow of nitrogen, 28 ml./min.; pressure of nitrogen, 36 cm. Hg

Curve B

The separation of aromatic hydrocarbons

Column length, 4 ft.; stationary phase, benzyldiphenyl; temperature, 100° C.; rate of flow of nitrogen, 42 ml./min.; pressure of nitrogen, 42.6 cm. Hg

FIG. 10. THE SEPARATION OF ALKYL CHLORIDES, AND OF ALCOHOLS



Curve A

The separation of alkyl chlorides

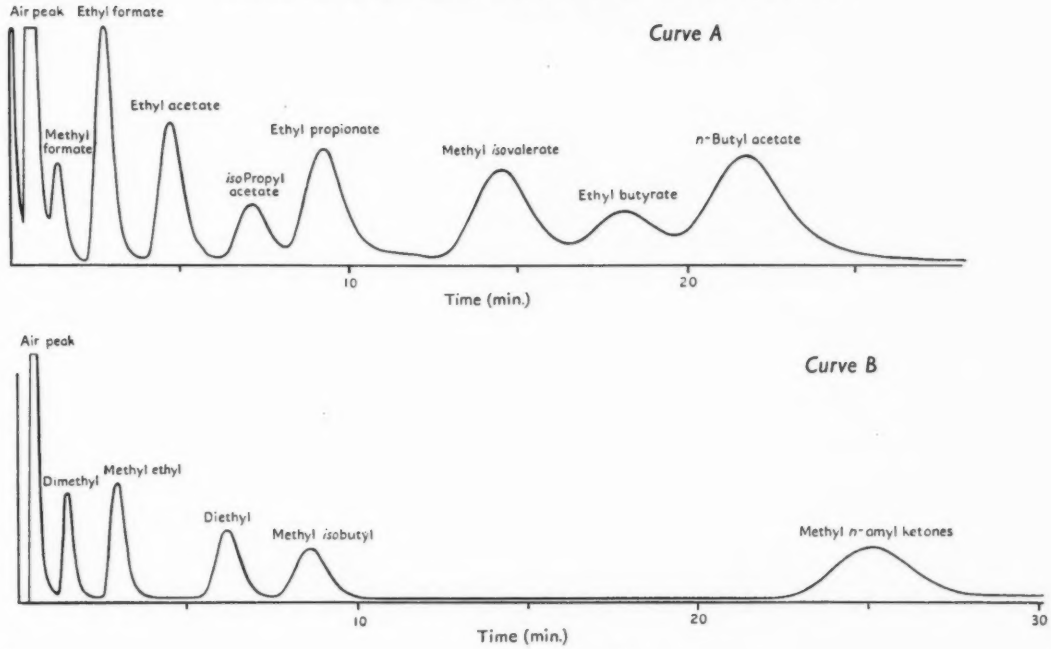
Column length, 4 ft.; stationary phase, benzyldiphenyl; temperature, 100° C.; rate of flow of nitrogen, 11.8 ml./min.; pressure of nitrogen, 12.5 cm. Hg

Curve B

The separation of alcohols

Column length, 4 ft.; stationary phase, benzyldiphenyl; temperature, 100° C.; rate of flow of nitrogen, 12 ml./min.; pressure of nitrogen, 13 cm. Hg

FIG. 11. THE SEPARATION OF ESTERS, AND OF KETONES



Curve A

The separation of esters

Column length, 4 ft.; stationary phase, benzyldiphenyl; temperature, 78.6° C.; rate of flow of nitrogen, 36.4 ml./min.; pressure of nitrogen, 27 cm. Hg

Curve B

The separation of ketones

Column length, 4 ft.; stationary phase, paraffin wax; temperature, 100° C.; rate of flow of nitrogen, 24.8 ml./min.; pressure of nitrogen, 17.8 cm. Hg

of the level of $\frac{1}{16}$ μg. of amyl alcohol/ml. of nitrogen being detectable. A schematic diagram of the complete apparatus is shown in fig. 8. As with the titration apparatus the whole procedure is automatically controlled once the column has been loaded and the nitrogen turned on.

The following figures show some of the separations obtainable with this apparatus. Figure 9, curve A, gives the separation of a series of aliphatic hydrocarbons from *n*-pentane to *n*-octane, the complete analysis taking only 34 minutes, the stationary phase on the column being liquid paraffin at a temperature of 78.6° C. The total load placed on the column is of the order of 2 mg. The separation of aliphatic hydrocarbons with their numerous isomers is a notoriously difficult and time-consuming process by the conventional method of analytical distillation. The use of gas–liquid chromatography enables a great deal of information to be obtained rapidly and easily, together with fairly unambiguous identification of most peaks. Indeed, by the use of long enough columns almost any degree of separation is obtainable. The first peak shown on the diagram is that due to the oxygen, argon, etc., in the small volume of air drawn on to the column in loading it, and is very useful for marking the time at which the nitrogen front passes out of the column. Figure 9, curve B, shows the separation of aromatic hydrocarbons, using a column with a stationary phase of the high boiling aromatic hydrocarbon, benzyldiphenyl. The presence of a small amount of impurity running between benzene and toluene is clearly demonstrated.

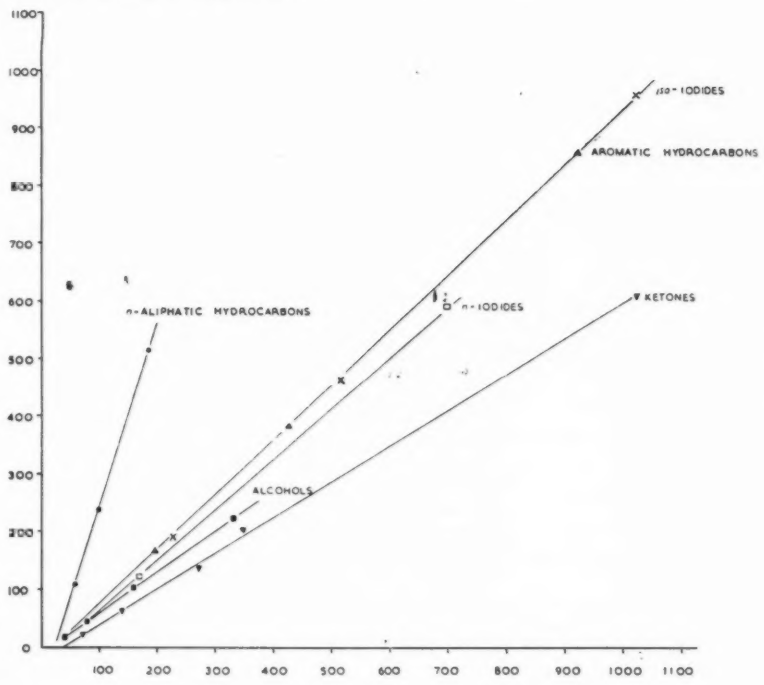
Figure 10, curve A, shows the separation of a series of alkyl chlorides from *iso*propyl to *iso*amyl chloride; total time of analysis, 22 minutes. Curve B shows the separation of alcohols from methyl to *n*-amyl alcohol. In this case ethanol and *iso*propanol are not completely separated; total time of analysis, 32 minutes.

Figure 11, curve A, shows the separation of esters ranging from methyl formate to *n*-butyl acetate; time of analysis, 26 minutes. Curve B shows the separation of a series of ketones, all being completely resolved.

It has already been pointed out that a great deal of useful information can be obtained by studying the behaviour of variously substituted compounds on two or more stationary phases where solution forces other than van der Waals forces may be involved. A further example of this is shown in fig. 12, on which the retention volumes of various homologous series in liquid paraffin as the stationary phase are plotted against retention volumes in the aromatic stationary phase, benzyldiphenyl. It can be seen that *n*-aliphatic hydrocarbons, aromatic hydrocarbons (aliphatic chlorides and bromides lie between and have been omitted for reasons of clarity), *n*-aliphatic iodides, alcohols and ketones all lie on straight lines of different slope, the decreasing slope indicating increasing interaction with the aromatic stationary phase. A more

detailed study of the series $C_5H_{11}—X$, where X was varied, showed that the interaction with the aromatic stationary phase increased in the following way: $X = H < Cl < Br < I < NH_2 < NO_3 < OH < COCH_3 < CN$. So a similar graph of homologous series of these compounds in the two types of column would give a family of straight lines with slope decreasing in the order shown. The same series studied in the two columns, liquid paraffin and a poly ether ($—CH_2CH_2OCH_2CH_2—$), gave a quite different order, viz. $H < OCH_3 < Br . Cl < I < NO_3 < COCH_3 < NH_2 < CN < OH$. Here obviously hydrogen bonding is playing a greater part than in the previous series. These specific interaction forces allow one to define closely the nature of an unknown volatile substance by running it on two or more types of column and measuring its retention volumes. The point representing the two values on the appropriate graph may then fall on one of a known family of lines. This method of studying relative interaction forces is much easier with gas–liquid columns than with liquid–liquid columns as there is only one phase, the liquid phase, in which the interaction can take place, and so the experimental data are more easily interpreted.

FIG. 12. GRAPHICAL REPRESENTATION OF RELATIVE INTERACTION FORCES IN TWO TYPES OF STATIONARY PHASE, LIQUID PARAFFIN AND BENZYLDIPHENYL



Abscissae: retention volume in benzyldiphenyl
Ordinates: retention volume in paraffin

To conclude, gas–liquid chromatography provides high efficiency columns with rapid flow-rates of the mobile phase so that analyses may be carried out rapidly and can provide detailed information on the structure of unknown substances. The technique should be capable of extension to all those substances capable of being distilled even at pressures of only a few millimetres of mercury. Its advantages over analytical distillation in terms of time, expense, and amount of quantitative data provided are enormous and in time this technique should completely replace analytical distillation.

* * *

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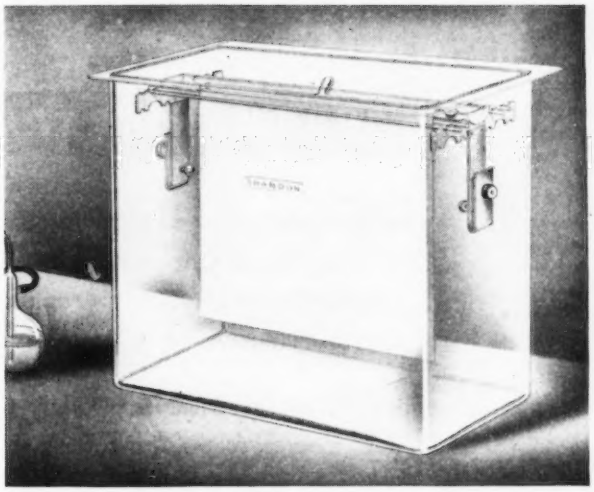

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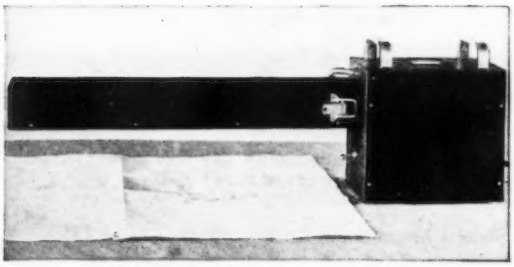

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PRACTICAL ASPECTS OF PAPER CHROMATOGRAPHY

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1 Methods of preparing paper chromatograms
  a Quantities of substances to be analysed
  b Time of development
  c Upward and downward development
2 Apparatus
  a Chambers
  b Troughs
  c Clips
  d Drying cabinet
  e Pipettes
  f Sprayers
  g Ultra-violet lamps
3 Materials
  a Paper
  b Solvents and solvent systems
4 Location of spots and identification
References

This article is intended mainly for those who are not very familiar with the methods of paper chromatography. So much has already been written about the subject that it will be treated only briefly here, but certain aspects of special concern to beginners will receive some attention. For further information, a number of books are now available to which the reader is referred. Those by Balston & Talbot (1952), Cramer (1953) and Block, Le Strange & Zweig (1952) deal specifically with paper chromatography. Two others deal with chromatography in general, but contain much of relevance to paper chromatography, namely Lederer & Lederer (1953), which can be highly recommended, and Brimley & Barrett (1953)[1], which is confined to practical aspects. Of the innumerable articles on the subject those by Dent (1951) and Stepka (1952) may be mentioned as being of interest to workers in the medical field.

1. Methods of Preparing Paper Chromatograms

Briefly, in paper chromatography, a small quantity of solution is placed near one end of a strip of filter-paper, and allowed to dry. The edge of the paper nearer the position of the drop is then brought in contact with a liquid which then flows along the paper. The whole process takes place in a closed system. After a certain time the paper is removed and dried. Under suitable conditions, the components of the original solution will have migrated at different rates from each other and so become separated. If colourless, they may be located as coloured spots by suitable reagents, or by other means described below.

The ratio of the distance moved by a given substance from the origin to the distance of the liquid front from the origin is known as its "R_F value". If the liquid has run off the paper the R_F value may be expressed relative to that of a control substance. (A simple elastic device for reading off R_F values directly has been described by Phillips, 1948.)

The process of chromatographic separation is known as "development" and this term should not be used for the subsequent procedure for locating the spots. The liquid used for development is sometimes referred to as the "mobile phase". This is usually an organic solvent saturated with water. The "stationary phase" is water saturated with the organic liquid, and is considered to be held stationary within the paper.

A large number of chromatograms may be developed side by side on a wide strip of paper by placing the solutions along a line parallel to an edge and sufficiently spaced (2–3 cm. apart) so that one chromatogram does not interfere with the next. In working with complex mixtures, it may happen that no single solvent system will be found that is capable of separating all the constituents. An improved and sometimes complete separation is achieved by the two-dimensional method. In this method the solution is placed near a corner of a sheet of paper. Chromatography is then carried out as described above. After development the sheet is dried. At this stage the constituents of the original mixture are distributed along a line, as in the strip method. If now the sheet is turned through a right angle and a second solvent allowed to flow through this line of spots, a further separation may be achieved. The resulting two-dimensional chromatogram will now appear as a series of spots distributed over the surface of the paper.

a. Quantities of Substances to be Analysed

The amounts and volumes employed are very small, but may vary considerably, depending on the limits which a given paper can deal with and on the sensitivity of the process for locating the spots. Common amounts are 1–10 μl. of solution containing 0.1–1% of each constituent, for one-dimensional separations. The two-dimensional method can deal with several times this quantity, but the volume in all cases should not normally be allowed to exceed 10 μl. If the solutions are so dilute that greater volumes are needed, successive small volumes may be placed at the origin, after each addition is dried. Relatively large amounts may be dealt with, by applying the solution along a line across a wide strip of paper. In this way milligram amounts may be separated, which may be sufficient for further investigation after extraction from the paper. Relatively large amounts of inorganic substances, such as salts and acids, in the test solutions will interfere with chromatography and must therefore be removed. This can be done chemically (e.g., precipitation of H_2SO_4 with $Ba(OH)_2$), by extraction of the substances with organic solvents or by electrolytic desalting (Consden, Gordon & Martin, 1947).

b. Time of Development

This may vary considerably, depending on many factors such as type of paper, length of paper, viscosity of solvent, the apparatus, the substances to be separated, and their amounts. Periods of an hour or two to several days are encountered. Overnight development can be very convenient, and conditions for this can usually be found after trial runs.

c. Upward and Downward Development

Development may take place in any direction, the two most usual being upward or downward. In upward development, which is the simpler method, the paper is supported at its

[1] Reviewed on p. 251 of this number of the Bulletin.—Ed.

upper end and hangs vertically with the lower edge dipping in the developing liquid. If the paper is turned to form a cylinder by stapling or stitching the edges, it will then stand in the liquid without a support. In downward development, the paper hangs vertically, with its upper edge held by a weight, such as a glass strip, in a trough which contains the developer. The paper must be prevented from touching the sides of the trough by a bar or rod over which the paper passes, and the origin of the solution should be below this bar. For a given solvent system, upward development is slower than downward. This is an advantage with rapidly moving solvents, since too fast a flow may not result in satisfactory chromatograms. On the other hand, the downward method will be preferable for slow-moving solvents. In the upward method, development of the chromatogram will stop when the solvent reaches the upper edge of the paper. Hence none of the components are lost, as can happen in downward development if the liquid is allowed to drip off the paper and substances of relatively high R_F value are present. However, for separating substances of low R_F value, which may require a long period of development, the downward method must be employed. Serrating the lower edge or fixing an absorbent pad to the lower edge are helpful devices for shortening the time and maintaining evenness of solvent flow in long development.

2. Apparatus

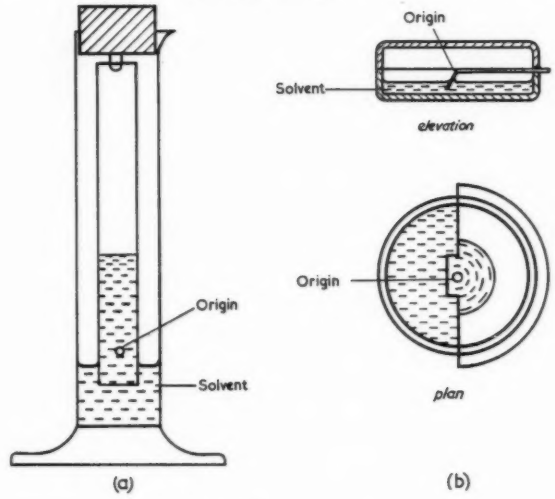
Successful paper chromatograms may often be produced in the simplest of laboratory apparatus, such as test-tubes or measuring cylinders. Filter-paper circles or semicircles held between two Petri dish lids also form a simple arrangement, but here, however, the separated components will appear as a series of arcs or circles. These methods are illustrated in fig. 1, and many other simple arrangements for small-scale chromatography can be devised. For larger-scale work, special apparatus, though not necessarily expensive, is required. These will be described in more detail below. Ancillary apparatus such as clips, drying cabinets, pipettes, sprayers and ultra-violet lamps will also be included. The basic requirements are containers which are reasonably air-tight, an atmosphere within the container saturated with the vapours of the components of the developing solvent system, and a fairly constant temperature, though the actual temperature is usually not important.

a. Chambers

i. *For one-dimensional separations.* Square or cylindrical all-glass containers of different sizes may be utilized. Two sizes of jar (21 in. high × 11 in. × 7 in. and 15 in. high × 8 in. × 5 in.)[2] are obtainable from Hospital and Laboratory Supplies Ltd., London, E.C.1. Battery jars serve admirably and can usually be obtained very cheaply in a variety of sizes. The top edges are ground flat so that air-tightness can be maintained by covering with a flat glass lid, aided if necessary by a coating of silicone grease. Saturation of the atmosphere is maintained by reservoirs (beakers or trays) within the chamber containing the components of the solvent system, or, where downward development is employed, the floor of the chamber may be covered with liquid. When volatile solvents are used, air-tightness and saturation of the atmosphere are particularly important, and it is a sound general rule, when using solvents which boil below 100° C., to place the paper in position within the closed chamber and to allow it to remain suspended in the vapours for an hour or so before introducing the developing liquid. This is done without removing the lid, if it is provided with a hole through which is inserted a tap funnel, fitted with a bung (cf. Isherwood & Jermyn, 1951 ; Hanes & Isherwood, 1949).

[2] 1 in. = 2.54 cm.—Ed.

FIG. 1. SIMPLE ARRANGEMENTS FOR PAPER CHROMATOGRAPHY



a: measuring cylinder
b: paper semicircle between two Petri dish lids

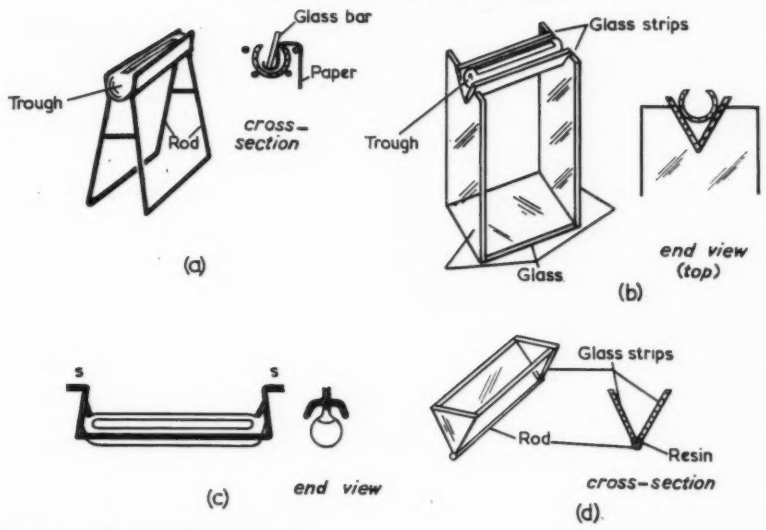
For upward development the paper may be conveniently suspended from a glass rod held in position against the walls by rubber or cork cushions. For downward development a trough mounted on a framework is required. The frames (see fig. 2) may be constructed of glass rod or sheets, or of material other than glass, such as stainless steel or certain synthetic resins, e.g., Lucite. (It must be borne in mind that many synthetic resins are attacked by some organic solvents or may evolve vapours that interfere with chromatography. These materials should therefore be tested beforehand in the solvent system which it is intended to use.) For certain purposes a framework independent of the chamber is an advantage (fig. 2a), since the placing of the paper in position or removing it after chromatography may be facilitated. With an independent framework standing in a tray of liquid, the chamber may be inverted over the framework and stood in the liquid, thus forming an air-tight system.

When relatively non-volatile solvents are used, slight leakage may be tolerated. Hence the trough provided with lugs (fig. 2c) may be mounted in slots ground into the tops of the walls of the container.

Besides glass, containers made of other materials may be used. The humble drain-pipe, originally mentioned by Consden, Gordon & Martin (1944) is still useful and cheap and can be obtained in a variety of sizes. However, since earthenware is somewhat porous it is advisable not to use the same drain-pipe for more than one solvent system.

ii. *For two-dimensional separations.* Two-dimensional separations are usually carried out with standard size sheets of paper (18½ in. × 22½ in.). Boxes are often constructed of plate glass in a framework which is made from a variety of

FIG. 2. TROUGHS AND FRAMES



a: glass rod framework and trough
(Cross-section shows arrangement for holding paper with glass bar.)
b: glass sheet framework and trough
(The sheets fit closely against the walls and floor of the container (not shown).)
c: trough with a sealed-on rod framework
(The side pieces (s) fit into grooves.)
d: trough constructed of glass strips and rod sealed with Araldite

materials, such as lead, Duralumin or wood (cf. fig. 3a). The wooden type coated with wax, described by Dent (1948), is obtainable commercially (Shandon Scientific Co., London, S.W.7). In the type of box illustrated in fig. 3a the trough and glass bars are mounted on a support, the whole being removable from the box to facilitate removal or insertion of paper. The reservoir containing the solvent system for saturating the atmosphere may be conveniently constructed from lead sheeting. The lid of the box is advantageously constructed with sloping sides to prevent condensed liquid dropping on to the paper.

Another type of box, which provides an air-tight liquid seal, is illustrated in fig. 3b. (This box has given good service for eight years.) The tray and outer cover are of tinned iron. A stainless steel framework for carrying the trough is bolted into the tray. Openings in the outside cover are provided for passing in gases if desired and for sucking off vapours. Other metals may be employed for making the boxes, but copper should be avoided, and where copper rivets have been used, or any parts have been brazed, the exposed portions should be coated with solder.

In some types of box, after chromatography, the wet paper is dried without removal. This is done by clipping the paper on to the bar or sandwiching it between a second bar and the existing one. The trough can then be removed, liquid in the reservoir, or reservoirs, sucked off, and hot air passed through the closed box, the exit fumes being drawn into a fume cupboard.

If boxes are made sufficiently wide to hold more than two troughs, more than two sheets of paper can be accommodated. A simple arrangement for simultaneously handling a large number of small squares of paper (20 cm. × 20 cm.) for upward two-dimensional development has been described by Datta, Dent & Harris (1950). This is a useful method for the rapid examination of a large number of clinical specimens (e.g., urines and sera), but more detailed investigations will require the larger apparatus.

Where large boxes unavoidably require moving about, it is useful to mount them on a platform with wheels. Boxes should also be supplied with a levelling device.

b. Troughs

Common laboratory apparatus, such as histological staining dishes, may be used for narrow strips. Hard glass troughs made to any specified size are obtainable from several glass manufacturing firms. The making of all-glass troughs in the laboratory is a rather laborious process. A number of methods have been described, but perhaps one of the least difficult is that of Atkinson (1948). Troughs may be very conveniently made by sealing strips of glass with resin-bonding materials such as Araldite (Aero Research Ltd., Duxford, Cambridge), provided that the resin withstands the solvents to be used. Troughs of this type will have a square or triangular cross-section (fig. 2d), the latter being an advantage because the supporting bars can be dispensed with.

Materials other than glass, such as porcelain, stainless steel and "polythene", are often used for making the troughs. Polythene troughs are easily made from 1–1½ in. piping, by cutting out a centre channel and sealing the open ends with flat polythene pieces. Glass rods are sealed on to form the bars. Polythene is resistant to most aliphatic solvents (e.g., alcohols, ketones, esters, hydrocarbons) but will soften with many aromatic liquids (e.g., phenol and coal-tar bases). Next to glass, stainless steel is probably the best material. Troughs to any specified size are obtainable from a number of manufacturers. The cheapest kind is constructed from sheeting (1 mm. thick) and the ends should be welded.

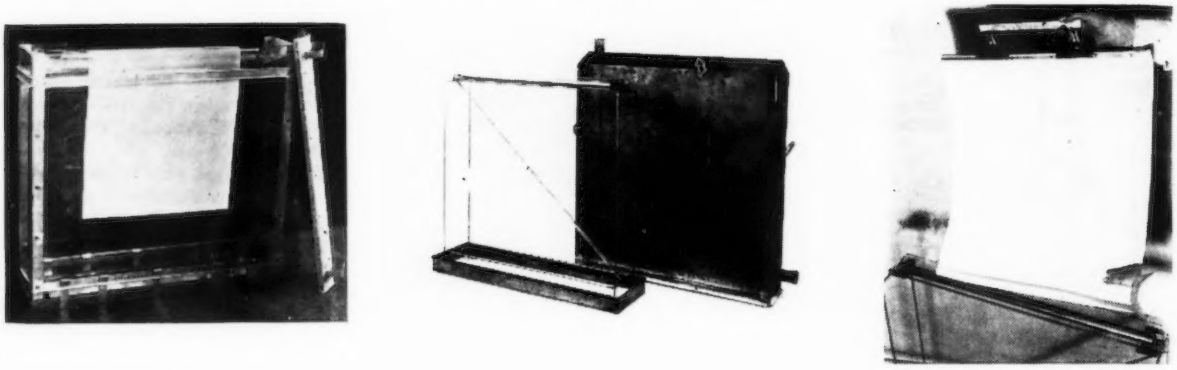
The dimensions of the troughs may vary very widely and their length will often be governed by the width of available containers. For two-dimensional work, a trough should have a capacity of 100–200 ml. and its length should be about 30 in. to enable it to accommodate the wider side of a standard sheet and leave room for a narrow strip.

c. Clips

In addition to common types of clip (e.g., "bulldog", which are useful for handling strips of paper), special clips are needed for wide sheets, especially when the paper is wet. One type, easily constructed from bulldog clips and tin strip, is illustrated in fig. 4a (see also fig. 3c). Another type specially designed for use in conjunction with the drying cabinet is illustrated in fig. 4b and described in section 2d.

Handling of paper. In general no difficulty is experienced in handling narrow strips, but large sheets of paper, particularly when wet, can present problems to the inexperienced.

FIG. 3. BOXES FOR TWO-DIMENSIONAL CHROMATOGRAPHY



a: glass box in Duralumin framework b: metal box c: showing insertion of paper into trough of metal box

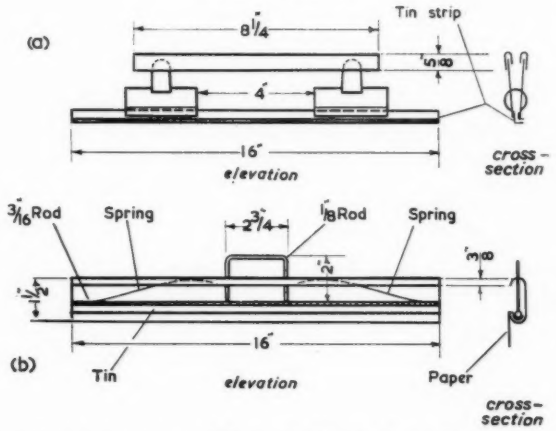
Some remarks have already been made concerning the handling of paper, but a few further points may be mentioned here, in connexion with two-dimensional downward development. Figure 3c illustrates a method of placing the paper in the trough which can be carried out by one person. The top edge of the paper is folded over (rubber gloves being worn) to make a crease ½–1 in. from, and parallel to, the edge. The fold may then be easily guided into the slot with a glass bar and pressed in as shown, the other edge being supported with a clip. For removal of the paper the above process is reversed. Before the clip is attached, any excess liquid is removed from the edge by blotting and, after removal, the edge which has been in the trough is blotted. Another method for removing the paper is to clip it on to the bar or rod support with two small clips, remove the bar from the trough and lift out the paper, taking care to prevent solvent on the edge which has lain in the trough from dripping on to the sheet. The bar holding the paper may serve as support in the drying cabinet (see section 2d), or the special clip may be attached to another edge (preferably not the one which is to go into the second solvent) and the bar removed.

When metal clips are being attached as weights to the lower edge in the cabinet, and this edge is to go into the second solvent, direct contact between metal and paper should be avoided by insertion of folded pads of filter-paper.

d. Drying Cabinet

A special apparatus is desirable for drying papers quickly at moderately low temperatures. It is particularly important that sheets should be thoroughly dried between the first and second developments in two-dimensional procedures. The main requirements for a drying cabinet are a good exhaust fan (e.g., speed, 2,800 rev./min.; air displacement, 420 cu. ft./min.)[3] and heating elements. The type illustrated in fig. 5 is constructed of metal, has a close-fitting door and accommodates two standard-size sheets. The lugs serve as supports for the special clips (fig. 4b) which carry the paper. When the door is closed, air is drawn through the slit at the top and heated by two 1-kilowatt heaters. It is advantageous to be able to vary the speed of the fan and to regulate the heaters, so that wet and therefore fragile paper can at first be dried gently. To prevent undue movement the paper is weighted at the bottom. Three-inch bulldog clips serve this purpose satisfactorily. For several reasons, it is better to arrange the draught to come through at the top of the cabinet rather than at the bottom. A glass window in the door is an advantage and a close-fitting glass plate at the back will facilitate cleaning, since the cabinet can also be used as a fume cupboard when the paper is being sprayed. The time of drying will, of course, depend on the solvent, ranging in this type of cabinet from 5–10 minutes for the lower fatty alcohols, to about 90 minutes for the cresols. The temperature within the cabinet does not rise above 50° C.

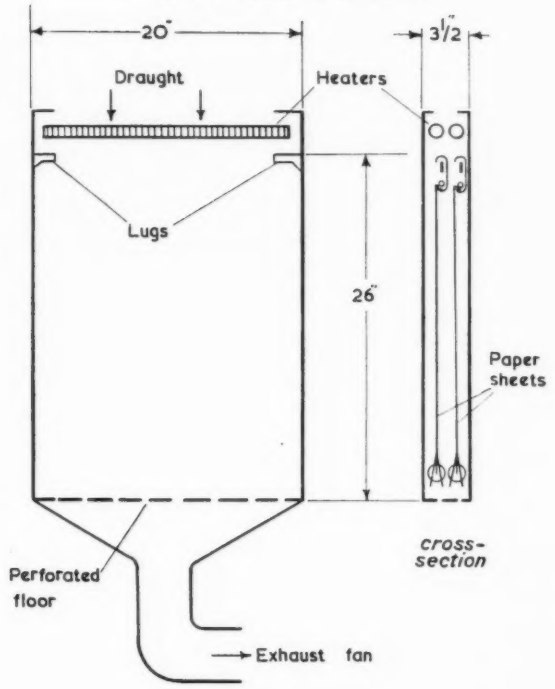
FIG. 4. CLIPS



a: apparatus constructed from "bulldog" clips with tin sheet soldered on
(For clarity, the cross-section shows clips partly open.)
b: special clip for use in drying cabinet
(In front elevation, clip is shown partly pulled out.)

[3] 1 cu. ft. = 0.02832 m.³—ED.

FIG. 5. DRYING CABINET



e. Pipettes

Micropipettes delivering up to 20 μl. are most commonly used. Where great accuracy is not required, a 0.1 ml. graduated blood pipette, marked in microlitre divisions, is suitable if cut to a convenient length (e.g., 12 cm.) which includes the bevelled end. Pipettes may be made from capillary tubing, and lengths with the end drawn to a point may be roughly calibrated by transferring known volumes from a blood pipette, or more accurately by weighing amounts of water between two marks. Uniform-bore tubing, which is manufactured commercially, is very convenient for making pipettes. Thus, 0.6 mm. bore is suitable for delivering multiples of 5 μl. (5 μl. corresponding to a length of about 1.5 cm.), and 0.23 mm. bore for multiples of 1 μl. (1 μl. corresponding to a length of about 2 cm.). Divisions should be marked by etching with hydrofluoric acid, not by scratching with a diamond. Pipettes for delivering single known volumes can be constructed by sealing a short piece of capillary tubing on to an ordinary piece of tubing, or by blowing a bulb above a short length of capillary tubing. By dipping the tip into the solution, the liquid will rise in the capillary and stop at the wide part. By bringing the tip of the pipette into contact with the paper the solution will flow out. The ends of pipettes should be drawn out and the tips flattened by rubbing on a fine sharpening stone. For highly accurate delivery, the Agla micrometer syringe (Burroughs Wellcome & Co., London, N.W.1) is suitable. It is important that pipettes should be kept scrupulously clean and cleaned immediately after use. They may conveniently be kept standing in distilled water when not in use.

f. Sprayers

It is usual to spray on to the paper the reagent for revealing the spots, although dipping is sometimes employed. A sprayer should deliver a stream of finely dispersed droplets and one should be able to cover the area of the paper rapidly and evenly. Throat sprayers have been used and there are many types of all-glass sprayers, some of which are obtainable commercially.

g. Ultra-Violet Lamps

Fluorescence in ultra-violet light or quenching of background fluorescence is exhibited by many compounds and this behaviour is sometimes very sensitive for locating spots on chromatograms. For this reason an ultra-violet lamp will often be very useful. The Hanovia Model XI fitted with a Woods glass filter is a high-pressure mercury type transmitting a range of 3,000–4,000 Å with a peak intensity at 3,660 Å. For lower wave-lengths, a low-pressure mercury arc lamp fitted with a Chance OX7 filter is manufactured by the same company (Hanovia Ltd., Slough, Bucks.). A description of this type of lamp has been given by Holiday & Johnson (1949).

3. Materials

a. Paper

The properties of different brands of filter-paper are dealt with fully in the books cited, particularly in Balston & Talbot (1952). Different brands vary in many respects, such as speed of development, wet strength, thickness, and impurities; and the choice will be governed by these factors. Many types of paper are used; perhaps the commonest are Whatman Nos. 1 and 4. Paper should be kept in a cool, clean place and direct contact with fingers avoided. Impurities vary considerably in amount between different brands, and those which can be present include inorganic salts, nitrogenous compounds (amino acids, peptides, ammonium salts), carbohydrates such as xylans and degraded cellulose, and metals. Some of these may interfere seriously with chromatography or with subsequent analytical procedures when the paper requires to be extracted, and the paper may require pre-treatment as, for example, in the chromatography of phosphate esters (Hanes & Isherwood, 1949), and quantitative determination of amino acids (Fowden & Penney, 1950). No paper appears to be free from traces of copper, zinc and other metals. These can interfere in chromatography, either by forming compounds with the substances to be separated (e.g., amino acids and phosphate esters) or by acting as catalysts in the oxidation of solvents, giving rise to dark-coloured viscous substances which distort the solvent flow, e.g., with phenol in an atmosphere of ammonia. This catalytic effect can be cut down by employing catalyst poisons in the atmosphere (coal gas, HCN, H_2S) or by dissolving reagents that form metal complexes in the solvent, such as ethylenediaminetetraacetic acid (Eggleston & Hems, 1952) or 8-hydroxyquinoline.

b. Solvents and Solvent Systems

Usually, organic solvents, which are not completely miscible with water, are shaken with excess of water in a separating funnel. The organic-rich phase is used for development, after it is ensured to be free from drops of water. The aqueous-rich phase and some of the organic phase may be used to saturate the atmosphere within the chamber. Solvents should be pure and may require pre-treatment. (Directions are given in the books cited in the introductory paragraph of this paper.) Water-saturated solvents should be stored in the dark. The

most common solvents are the lower alcohols up to C_5, aliphatic esters and ketones, phenol and the cresols and coal-tar bases (collidine and the lutidines). The latter may be obtained from the Yorkshire Tar Distillers, Barnsley, Yorks., and usually require pre-treatment to render them suitable for chromatographic work. Constant temperature is important for water-saturated collidine, since a relatively small rise in temperature results in the precipitation of water, which will distort the chromatogram. A useful precaution is to use it slightly undersaturated (*ca* 90%). Phenol and the cresols may be obtained very pure from Monsanto Chemicals Ltd., Ruabon, North Wales, and for most purposes require no preliminary purification. After saturation of phenol or cresol with water, coal gas may be bubbled into the solution for a short time. This helps to prevent the formation of deeply coloured oxidation compounds on standing. A stock of water-saturated phenol or cresol should also be prepared containing 0.1% (w/v) of 8-hydroxyquinoline.

Sometimes a mixture of more than one organic solvent saturated with water is used for development. It is important, in these cases, to adhere to definite proportions of each constituent whenever the solution is prepared. Occasionally, aqueous organic liquids completely miscible with water have been employed and here again the proportions are important.

The composition of an aqueous solvent may alter, owing to hydrolysis, esterification, and so forth. These mixtures may be used either freshly prepared or after ageing sufficiently to allow equilibrium to be reached, as in the case of aqueous esters and butanol–acetic acid–water mixture.

Choice of solvent is governed by many factors and is often arrived at empirically. The following general rules may be of guidance:

i. For an homologous series of solvents, the R_F value of a substance will vary inversely with the molecular weight of the solvent. Thus, if the R_F value is too high in one solvent, a lower homologue may prove satisfactory.

ii. The R_F value of a substance tends to increase with increase of water content of the solvent system. This effect may be achieved by adding more polar liquids to solvents before saturation with water. Examples are incorporation of ethanol or acetic acid with *n*-butanol for sugars, and 2:4-lutidine with *s*-collidine for amino acids.

iii. If the substance is a weak acid or base, suppression of ionization will tend to increase its R_F value and vice versa. For example, ammonia is used in the atmosphere when phenol is the developing solvent for protein hydrolysates. This retards the dicarboxylic amino acids and hastens the basic amino acids.

iv. Substances will be separable if their R_F values differ by 10% or more. If substances have high R_F values (0.8 or more) in a given solvent, separation is likely to be more difficult than in a solvent in which their R_F values are lower.

4. Location of Spots and Identification

i. *Location of spots.* In general, the methods for revealing the position of colourless substances on chromatograms fall into four categories: (i) spraying or dipping with reagents that form coloured compounds, (ii) examination in ultra-violet light, (iii) microbiological methods and (iv) employment of radioactive tracers. It is outside the scope of this article to discuss the last method; neither will any further remarks be made about category (ii).

The first method is the most common, and the number of reagents which have been described for all classes of compounds and for specific chemical groups is already legion. It is impossible to discuss these in detail here. Two points, however, are considered worthy of mention. Often, after the application of the reagent, the chromatogram requires heating for the reaction to occur. This is usually carried out in an oven, but it may not be realized that many reactions require the presence of water. Therefore heating in steam may often considerably enhance the sensitivity. Since many of the colours produced after the reaction fade or become obliterated by the formation of background colour, it is usual to ring the spots in pencil in order to obtain a permanent record.

In the microbiological method, a biologically active substance or mixture is analysed by paper chromatography and the strip of paper is then laid in contact with agar seeded with the appropriate organisms. After incubation, the positions of the substances are revealed by inhibition or growth promotion of the organism. This method, first reported by Goodall & Levi (1946) has proved of great value in the study of many antibiotics and growth promoters, and is often extremely sensitive (e.g. Woodruff & Foster, 1950). Useful accounts of this method have been given by Proom & Woiwod (1953) and by Forsyth (1953).

ii. *Identification.* Position on the chromatogram, colour reactions with chosen reagents, and behaviour in different solvent systems are aids to identification. For such tests, a control mixture of known substances should always be developed adjacent to the test substance in one-dimensional chromatograms. Strong confirmation of the identity of two substances is obtained by the mixture behaving as a single spot in a number of different solvents.

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QUANTITATIVE DETERMINATION OF AMINO ACIDS

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1 Paper chromatography
  a Two-dimensional chromatography
  b One-dimensional chromatography
  c Miscellaneous techniques
2 Column chromatography
  a Starch columns
  b Ion-exchange columns
  c Column chromatography of dinitrophenyl-amino acids
References

During the past decade chromatography in its various forms has revolutionized the methods used in the quantitative analysis of the amino acids given on hydrolysis of peptides and proteins. We are, indeed, now in the happy position of having routine procedures which, at a cost of only a few milligrams of material, will give results of a sufficiently high order of accuracy for all the amino acids except tryptophan. This is certainly the case where the object is to assess nutritive value, but if the data are to be used as a guide in solving problems of internal structure, the limitations imposed by the size of the peptide or protein molecule concerned must be kept in mind (Tristram, 1949). The literature dealing with the quantitative aspects of chromatographic analysis is growing steadily month by month and, as the theory of " partition " chromatography is dealt with elsewhere[1], this short review is limited to a brief description of recent methods and results. No attempt has been made to discuss the finer points of analysis, and the choice of topics has been dictated largely by the personal experience of the writer.

1. Paper Chromatography

Paper chromatography was first designed, and is still widely used, for qualitative analysis. Simple in operation, and applicable to minute amounts of material, the urge to make it a medium for quantitative analysis was a logical development of immediate appeal. Many of the early attempts to do this (reviewed by Martin, 1948; Strain, 1950; Clegg, 1950), in which the workers concerned claimed a degree of accuracy that was probably not justified, met with indifferent success in other hands. Progress, however, was rapid, yet when Moore & Stein (1952) discussed the position a little later they expressed the opinion that more experience would be needed before the newer methods then coming into vogue could be fully assessed. Since then the focus of research has shifted somewhat, and we are now witnessing a slower but more systematic approach in which the emphasis is directed towards a good separation into small compacted spots and reproducibility of results. Several of the more recent investigations on the separation and estimation of amino-acid mixtures on one-dimensional chromatograms are indeed very encouraging, but Moore and Stein's dictum still holds. Except in one particular case it has yet to be demonstrated that the new procedures can be applied with the necessary degree of accuracy and reliability to the mixed products of protein or peptide hydrolysis.

a. Two-Dimensional Chromatography

Fowden (1951) locates the spots by fluorescence and estimates the amino acids with ninhydrin after elution of the paper segments concerned. He showed that the ammonia content of the paper was often the cause of high and variable blank readings, and he removes this content prior to elution by suitable treatment of the segments with sodium hydroxide. The procedure of Boissonnas (1950) is somewhat similar though the details of technique are different. Levy & Chung (1953) suggest new buffered solvent systems that give sharper and less irregular-shaped spots than hitherto. None of these elution procedures seem to have been applied to hydrolysates of proteins or peptides. Thompson, Zacharius & Steward (1951) develop the chromatogram under anaerobic conditions with a solution of ninhydrin which does not contain a reducing agent. The blue colour of the spots is then eluted for photometric measurement. Thompson & Steward (1951) claim that the procedure, designed primarily for the estimation of amino acids in plant extracts, gives good recoveries under rigidly standardized conditions, but their analysis of a casein hydrolysate is not impressive. It is the opinion of the present reviewer that, for quantitative analysis, two-dimensional chromatography is unlikely to hold its own in competition with the slightly more elaborate one-dimensional procedures mentioned below.

b. One-Dimensional Chromatography

Several new procedures have been put forward recently, which differ in the solvent systems employed, the colour reagents used and the conditions under which the colour is developed. All of them give a much better separation of the amino acids than hitherto, and the authors prefer to use the ninhydrin-developed spots rather than an elution method for the estimation. Fisher, Parsons & Morrison (1948) measure the area of the spots and claim a high precision (2–4%) with simple mixtures of amino acids, but have made no further report. Direct photometry has been used in the more recent investigations, applied either by scanning an entire strip of fixed width (Bull, Hahn & Baptist, 1949) with a densitometer, or more rapidly, though less accurately, by measuring the maximum density of each spot. Block (1948, 1950) runs a series of six chromatograms with various solvents. On one of these the tyrosine and histidine are developed with diazotized sulphanilamide; on another the proline and hydroxyproline with isatin (Acher, Fromageot & Jutisz, 1950); on a third the methionine with platinic iodide in acetone; and on the other three the remaining amino acids with ninhydrin, which is allowed to act for 18–28 hours at room temperature. The photometry is by maximum density. The method is designed for the rapid analysis of food proteins and may well be suitable for the purpose. McFarren (1951) obtains good separation on a series of buffered chromatograms run with various solvent systems buffered at the corresponding pH. The papers are dried at 90–95° C., and the colour

[1] See Williams, p. 165 of this number of the Bulletin.—Ed.

developed with ninhydrin for 10 minutes at 90° C. It is claimed that the spots are suitable for direct photometry, but no analytical data have been published. Patton & Chism (1951) use three solvent systems—phenol, ethanol and lutidine—all with water, and develop in the dark at room temperature for 18 hours with ninhydrin dissolved in 95% ethanol. The amino acids are determined photometrically against known standards, and in each case a series is run so that comparison of the known and unknown is made at the optimal concentration. Various points of technique are discussed in a more recent paper (Salander, Piano & Patton, 1953) and a high degree of reproducibility is claimed, but supporting evidence with protein hydrolysates has not yet been published. Redfield & Barron (1952) achieve a complete separation of all the amino acids of a protein hydrolysate by using a number of simple alcohol solvents. The chromatograms are cut into strips for photometric scanning after treatment with ninhydrin (isatin in the case of proline). The ninhydrin-sprayed strips are air-dried for 30 minutes and then kept for 10 minutes at 90° C. in the presence of water vapour. The coefficient of variation for a single determination is stated to be ± 4.36%. Redfield and Barron's analysis of bovine serum albumin (Table I) compares very favourably indeed with that of Stein & Moore (1949), who used a starch column.

TABLE I. AMINO-ACID COMPOSITION OF BOVINE SERUM ALBUMIN

Amino acid	Grams of amino-acid residue/100 g. of protein	
	Paper chromatography *	Starch column †
Glycine	1.42	1.38
Serine	3.67	3.51
Threonine	5.04	4.95
Alanine	5.41	4.99
Phenylalanine	5.69	5.87
Leucine	10.25	10.58
Isoleucine	2.24	2.25
Aspartic acid	9.15	9.44
Glutamic acid	15.25	14.49
Valine	4.68	5.01
Methionine	0.74	0.71
Tyrosine	4.64	4.56
Histidine	3.63	3.54
Proline	3.68	4.00
Arginine	5.38	5.29
Lysine	11.25	11.25
Cyst(e)ine	5.5	5.54
Tryptophan	0.53‡	0.53‡
Total	98.1	97.9

* Redfield & Barron (1952)
† Stein & Moore (1949)
‡ Brand (1946)

c. Miscellaneous Techniques

Kemble & Macpherson (1954), seeking for a method of determination less fickle than ninhydrin colorimetry, measure the CO_2 evolution on oxidation with chloramine-T (in the presence of formalin to suppress ammonia interference) in a Warburg respirometer. The procedure has so far been applied only to the monoamino-acid fraction obtained by ionophoresis. A 9 in.[2] wide strip of Whatman 3 MM paper will separate at least 2 mg. of each amino acid, sufficient to give a maximum reading on the manometer. The modified isotope method of Keston, Udenfriend & Levy (1950), using chromatography and labelled tracer amino acids, should be capable of high accuracy, but it is too laborious for general use. The radiochemical technique of Blackburn & Robson (1953), in which the amino acids separated by chromatography are converted to their copper complexes with copper phosphate containing ^{64}Cu, should be explored further; an accuracy of ± 3% is claimed. The circular paper chromatography introduced by Rutter (1948, 1950), for which the advantages of simplicity of apparatus, speed and reproducibility are claimed, has been explored by many workers. It has yet to prove its worth as a method of quantitative analysis. The more recent procedures of Matthias (1954) and Schwerdtfeger (1954) have most fascinating possibilities.

2. Column Chromatography

a. Starch Columns

The success of paper chromatography naturally suggested that fractionation on columns of powdered cellulose might be of use (Peterson & Reineke, 1949; Synge, 1951). Stein & Moore (1948, 1949, 1950), however, demonstrated that a better resolution could be obtained with starch, which they made the vehicle for a very effective method of quantitative amino-acid analysis. An amount of material representing about 3 mg. of protein is transferred to a 30 cm. column, which is then developed with two different solvent mixtures in succession. The effluent is collected in a series of tubes with the aid of a fraction collector, and the contents of each tube are evaluated from the colour produced on treatment with their special ninhydrin reagent. The effluent curve is made up of a series of peaks which represent, except in two cases, the individual amino acids. The exceptions are pairs of products which can, in turn, be separated by further treatment on the column with appropriate solvents. The recoveries are quantitative (100 ± 3%) except for cyst(e)ine. In protein hydrolysates, of course, tryptophan is not estimated as it is largely destroyed. Excellent analyses have been published for bovine serum albumin (Table I) and β-lactoglobulin (Stein & Moore, 1949), haemoglobin from normal negroes and from negroes with sickle-cell anaemia (Schroeder, Kay & Wells, 1950) and the oxytocic hormone (Pierce & du Vigneaud, 1950). As all the amino acids, other than those stated, can be estimated on the sample of the hydrolysate with the minimum of chemical manipulation the method, when first introduced, seemed to be the ideal one for the routine analysis of proteins. More recent experience, however, suggests that ion-exchange columns are even more effective for this purpose.

b. Ion-Exchange Columns

Moore & Stein (1951) use the sulphonated polystyrene resin, Dowex 50, in the sodium form, and find that a 100 cm. column has a higher resolving power and greater capacity than a similar one of starch. Elution of the amino acids with aqueous buffers in the range from pH 3 to pH 11 gives an effluent curve on which each of the more commonly occurring amino acids appears as a separate peak. The recoveries of the neutral acids (except methionine, cystine and tryptophan, which are mentioned later), and the dicarboxylic acids, are quantitative (100 ± 3%), but for the basic acids they prefer to use a shorter column (15 cm.) and solutions

[3] 1 in. = 2.54 cm.—Ed.

TABLE II. AMINO-ACID COMPOSITION OF INSULINS FROM DIFFERENT SOURCES (Harfenist, 1953)

Amino acid	Number of residues/mole of insulin			
	Pig	Sheep	Ox	
	Found	Found	Found	Computed from Sanger, Thompson & Tuppy (1952)
Aspartic acid	2.93 ± 0.05	3.00 ± 0.02	2.89 ± 0.06	3
Threonine	1.77 ± 0.10	0.96 ± 0.04	0.97 ± 0.03	1
Serine	2.76 ± 0.11	2.06 ± 0.04	2.89 ± 0.01	3
Glutamic acid	7.05 ± 0.07	6.95 ± 0.01	6.74 ± 0.11	7
Proline	1.06 ± 0.03	1.10 ± 0.02	1.07	1
Glycine	3.97 ± 0.05	4.69 ± 0.04	3.95 ± 0.08	4
Alanine	2.16 ± 0.10	2.99 ± 0.02	2.95 ± 0.02	3
Cystine	3.03	3.10	2.91	3
Valine	3.70 ± 0.09	4.78 ± 0.03	4.73 ± 0.01	5
Isoleucine	1.54 ± 0.04	0.69 ± 0.02	0.66 ± 0.02	1
Leucine	6.00 ± 0.10	5.98 ± 0.18	5.85 ± 0.18	6
Tyrosine	3.99 ± 0.11	4.08 ± 0.11	3.98 ± 0.10	4
Phenylalanine	2.99 ± 0.08	3.06 ± 0.08	2.98 ± 0.07	3
Histidine	1.96 ± 0.10	1.90 ± 0.03	2.00 ± 0.06	2
Lysine	0.98 ± 0.01	1.02 ± 0.03	1.01 ± 0.04	1
Arginine	0.89 ± 0.03	0.94 ± 0.02	1.02 ± 0.05	1
Ammonia	5.88 ± 0.35	6.14	5.65 ± 0.24	6

buffered below pH 7. The completeness of the procedure is illustrated by the recent work of Harfenist (1953) on insulin from three different species (Table II). It will be noted that her results for the ox variety are in excellent agreement with those computed from Sanger's work on the amino-acid sequences in this protein (Sanger, Thompson & Tuppy, 1952).

These studies of Harfenist have brought out clearly the need in certain cases to extend the period of hydrolysis with 5.7 N-HCl beyond the usual 20–24 hours. To obtain a good recovery of isoleucine and valine she found it necessary to carry on for 96 hours, thus suggesting that the peptide linkage in the sequence isoleucylvaline, shown to be present in this protein by Sanger & Thompson (1953), is, like that of valylvaline (Synge, 1945), resistant to hydrolysis because of steric considerations. Her finding that tyrosine is the only other amino acid besides serine and threonine (Rees, 1946) to undergo any appreciable decomposition during this longer period of hydrolysis may have important applications.

Insulin, however, has a somewhat unusual composition, in that it contains neither tryptophan nor methionine, so that Harfenist was able to compute the cystine content without equivocation from the value for total protein-S. As all of these three particular amino acids are of importance in nutrition, and are also prone to decomposition during acid hydrolysis in the presence of extraneous carbohydrate material, it is gratifying to note that they are receiving close attention in the comprehensive investigation on the free and combined amino acids of foodstuffs that is being undertaken by Dr Stanford Moore and Professor E. J. Bigwood in the latter's laboratory at Brussels.

To estimate cyst(e)ine they treat the material before hydrolysis with performic acid so as to oxidize the residues of cyst(e)ine to those of cysteic acid (Sanger, 1949). After hydrolysis the cysteic acid can be estimated quantitatively on the column (Schram, Dustin, Moore & Bigwood, 1953). The small amount of decomposition undergone by methionine on acid hydrolysis (Moore & Stein, 1951), which is due largely to conversion to the sulphoxide, is enhanced by the presence of carbohydrate. The sulphoxide can, nevertheless, be estimated on the column and so included in a provisional estimate of the methionine (Schram, 1952). Dustin, Schram, Moore & Bigwood (1953) find that, if hydrolysis is conducted under conditions of considerable dilution (5-10 mg. of protein/200 ml. of 6 N-HCl), the amino acids (except tryptophan) undergo but little decomposition even in the presence of as much as a hundredfold excess of carbohydrate. Such a sparing action of dilution was first noted by Jacobsen (1949). All of these findings augur well for the future. Tryptophan, however, still presents difficulties which have yet to be overcome, as an attempt to estimate it on a column after alkaline hydrolysis was not successful (Drèze, 1952).

It will be seen that the Moore and Stein procedure, with the few modifications suggested by Harfenist and the Brussels school, should enable the amino-acid composition of proteins, and even of crude protein foodstuffs, to be estimated with a considerable degree of confidence. Some typical analyses of the latter type of material by Dustin *et al.* (1953) are given in Table III. The low over-all recovery is almost certainly due to the presence therein of nitrogenous products that are not amino acids. The procedure is clearly giving important results, but it should be emphasized that rigid control of technique at each stage is necessary, and that the full procedure is far more time-consuming than might be expected. The Moore and Stein ninhydrin reagent calls for purified ninhydrin, which—in England—is expensive. The use of the ninhydrin procedure of Troll & Cannan (1953) may therefore be worth exploring, as this gives satisfactory results with the commercial product. The colour is developed in the presence of pyridine, which may give a high blank unless it is first distilled in the presence of a small amount of bromine (2 ml./l.).

TABLE III. AMINO-ACID COMPOSITION OF VARIOUS FOODSTUFFS (Dustin, Schram, Moore & Bigwood, 1953)

Amino acid	N as % of total N		
	Barley grain	Hay	Linseed cake
Aspartic acid	3.9	6.0	6.2
Glutamic acid	12.4	6.0	11.0
Glycine	5.0	5.7	6.6
Alanine	4.3	6.0	4.6
Valine	3.5	3.8	3.5
Isoleucine	2.2	2.8	2.8
Leucine	4.5	4.7	4.3
Serine	3.1	3.0	4.0
Threonine	2.7	2.9	3.0
Cystine	1.8	0.9	1.4
Methionine	0.9	0.9	0.7
Proline	7.1	4.5	2.6
Phenylalanine	2.6	2.6	2.4
Tyrosine	1.5	1.3	1.2
Tryptophan	0.9	0.9	1.0
Histidine	3.4	2.5	3.0
Lysine	4.0	5.4	4.0
Arginine	9.7	9.2	19.1
Ammonia	14.0	9.4	12.4
Total	87.5	78.5	93.8

c. Column Chromatography of Dinitrophenyl-Amino Acids

The dinitrophenyl- (DNP-) amino acids are highly coloured and lend themselves readily to separation by liquid–liquid chromatography. In the well-known method of Sanger for the identification and estimation of *N*-terminal residues, the protein (or peptide) is treated with fluorodinitrobenzene (FDNB) and the DNP-amino acids given on subsequent hydrolysis are separated on columns (Sanger, 1945; Porter & Sanger, 1948) or buffered columns (Blackburn, 1949) of silica gel. As an extension of this procedure Mills (1952) has applied FDNB under specified conditions after hydrolysis of the protein to convert all the amino acids into their DNP-derivatives; these can then be separated with reasonable efficiency on a simple column of kieselguhr by developing with various solvent mixtures of methyl ethyl ketone and chloroform saturated with water. The resolution is not, at present, as high as that obtained by Moore and Stein on columns of starch or Dowex 50, but Mills' results for salmine, ox insulin and lysozyme compare favourably with those recorded in the literature. As the procedure is rapid in operation and calls for no special equipment, it may be well worth exploiting in studies of amino-acid sequences in peptides. Independently, and under different conditions, it has been used by Callow & Work (1952) for the estimation of certain of the amino acids given on hydrolysis of antibiotic peptides (licheniformins A and B) from *Bacillus licheniformis*.

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INORGANIC CHROMATOGRAPHY

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1 Ion exchange
2 Alumina
3 Selective reactants
4 Cellulose—general considerations
5 Schemes of analysis
6 Quantitative determinations
References

Knowledge of the potentialities of inorganic chromatography has been of recent growth, though interest in similar processes dates back to the middle of the nineteenth century. There has now been developed a variety of methods, both for analytical and preparative purposes. In addition to simplifying general analytical procedures, separations of inorganic substances have been achieved that hitherto were either impossible or extremely tedious by classical chemical methods. Some techniques are well suited for microchemical work, and for concentrating traces of material.

The chromatographic separation of inorganic substances has been investigated on (i) ion-exchange resins, (ii) supporting materials which act as good adsorbents, e.g., alumina, (iii) columns of selective reactants, e.g., 8-hydroxyquinoline, and (iv) cellulose columns. Remarkable progress has been made in the field of paper and cellulose chromatography, and this will form the major topic of this article.

The elution analysis method is most generally used in operating columns. This consists of putting the mixture to be separated, in a suitable solution, on the top of the column. A solvent is then used to irrigate the column in order to separate the constituents of the mixture into zones. As the zones emerge from the bottom of the column, they are collected in fractions. The Tswett method of extrusion has to be used if the zones are strongly held by the supporting material. Various types of eluting solvent are used, depending upon the constituents of the mixture to be separated, and the nature of the supporting material in the column.

Frontal analysis and displacement analysis have not been used very extensively, though both may take part in processes which appear to be elution analysis.

Detailed references are not given since these can be found in *Ion exchangers in analytical chemistry* (Samuelson, 1953) and in *Chromatographic methods of inorganic analysis, with special reference to paper chromatography* (Pollard & McOmie, 1953).

1. Ion Exchange

Since the early work on ion exchange with clays, and the well-known use of zeolites for water softening, new organic resins specially synthesized for cation or anion exchange have been widely used. There are two methods of using ion-exchange materials: (i) for the separation of a mixture of exchangeable ions, depending upon the differences in exchangeability of the ions, and (ii) for the separation of exchangeable ions from non-exchangeable ions. The former can be regarded as chromatography, while the latter is not.

In ion-exchange chromatography of inorganic cations, the mixture to be separated is placed on the top of the column and eluted with aqueous solutions containing a complex-forming reagent, which may act as a buffer solution. The control of pH of the eluting solvent is a very important factor for successful separations. The most outstanding examples of this technique are the separations of the rare earths and the transuranic elements, while of medical interest there is the isolation of radioactive tracers from urine.

The second method of using ion-exchange columns is probably the most widely used and, though not strictly chromatography, it can be used in conjunction with other chromatographic processes. It is important for the removal of interfering ions or for the concentration of traces of metals or anions, and has been used for the determination of ammonia in urine by separating the ammonia from interfering amino acids. An anion-exchanger is of value for removing chloride, phosphate, sulphate, etc., where these may interfere in analysis. The concentration of metal ions in natural waters is achieved by passing the water through a cation-exchange resin, when the cations are retained on the resin. After the resin is washed with metal-free distilled water, the cations are then desorbed with hydrochloric acid. In a similar manner, copper in milk, tin and copper and iron in beer, sodium and potassium in apple juice, have been separated from the bulk liquor.

2. Alumina

The revival of interest in chromatography during the late 1930's led Schwab and co-workers to investigate the separation of cations and anions on columns of alumina (Schwab & Jockers, 1937; Schwab & Ghosh, 1939). Aqueous solutions of ions are adsorbed on the top of the column, which is then irrigated with water, acid, or alkali; the orders of the common cations and anions from top to bottom of the column are as follows:

Cations

$$\left.\begin{matrix}Th\\Al\\U^{4+}\end{matrix}\right\},\ \left.\begin{matrix}Zr\\Cr\\Ce^{4+}\end{matrix}\right\},\ \left.\begin{matrix}Fe^{3+}\\Ti\\Ce^{3+}\end{matrix}\right\},\ Hg^{2+},\ UO_2,\ Pb,\ Cu,\ Ag,\ Zn,\ \left.\begin{matrix}Fe^{2+}\\Co\end{matrix}\right\},\ \left.\begin{matrix}Ni\\Cd\end{matrix}\right\},\ Tl^{+},\ Mn$$

Anions

$$OH^{-},\ PO_4^{3-},\ C_2O_4^{2-},\ F^{-},\ \left.\begin{matrix}SO_3^{2-}\\[Fe(CN)_6]^{4-}\\CrO_4^{2-}\end{matrix}\right\},\ S_2O_3^{2-},\ SO_4^{2-},\ \left.\begin{matrix}[Fe(CN)_6]^{3-}\\Cr_2O_7^{2-}\end{matrix}\right\},$$

$$\left.\begin{matrix}NO_2^{-}\\CNS^{-}\end{matrix}\right\},\ I^{-},\ Br^{-},\ Cl^{-},\ NO_3^{-},\ MnO_4^{-},\ ClO_4^{-},\ CH_3CO_2^{-},\ S^{2-}$$

Many separations can be achieved, but in general there is overlapping of zones, leading to difficulties in detection and in the use of the method for quantitative analysis. The order of separation is affected if complexing agents, e.g., tartaric acid, are introduced into the eluting solvent. Though the applications are limited compared with other supports like cellulose, successful use of alumina has been made for freeing inorganic solutions from certain trace impurities; quantitative methods for cobalt, cadmium, copper, iron, and lead have been reported. Hopf (1946) and Flood (1949) have used paper impregnated with alumina, which behaves somewhat similarly to a column of alumina.

3. Selective Reactants

A number of substances that react selectively to form definite chemical compounds have been utilized as supports. Among the most interesting have been columns of 8-hydroxyquinoline for the analysis of non-ferrous alloys, and violuric acid for the separation of the alkali metals, alkaline earth metals, copper, lead, and mercury.

Recently (Carrit, 1953) a column consisting of a solution of dithizone in carbon tetrachloride supported on granular cellulose acetate has been employed to concentrate trace metals in water. A sample of mineral water was passed through the column when the dithizonates of lead, zinc, cadmium, manganese, copper, and cobalt were formed and held on the column. The column was rinsed with metal-free water, eluted first with N-HCl to remove lead, zinc, cadmium, and manganese, and then with concentrated acid to remove copper and cobalt. The efficiency of this process was claimed to be better than by separate processes of liquid–liquid extraction, and concentration factors up to 1,000 have been achieved, though a factor of as much as 10,000 was thought to be practicable.

4. Cellulose—General Considerations

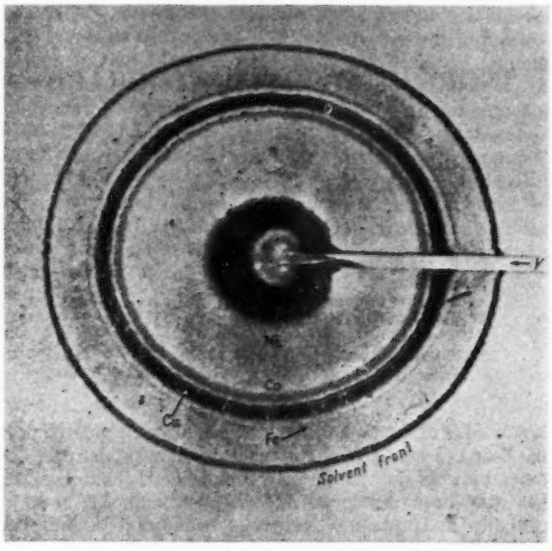
The simplicity with which the separation of inorganic substances can be achieved by partition chromatography on cellulose makes this method attractive for all types of qualitative and quantitative applications. The general procedure is not essentially different from that originally used by Martin and Synge for organic compounds, but the processes involved in the separation may not be solely partition. There is evidence of adsorption of the type associated with alumina, and even ion exchange involving the exchangeable hydrogen of the cellulose.

The quantities of material that can be separated range from 1 mg. to 0.15 g. on filter-paper of varying thicknesses, up to amounts of 0.5 kg. on columns of cellulose powder. The mixture to be separated is placed at the top of the column and then eluted with an organic solvent containing water, acid and perhaps a complex-forming reagent. Both upward and downward chromatography with paper strips are employed, and the Rutter disk method (fig. 1) can often be used for qualitative analysis. On the larger scale, columns of cellulose up to almost any size can be used, together with a suitable fraction collector.

Where there is a trace of metal compound in the presence of a large quantity of other material, it is often possible to separate and concentrate the trace, using a triangular-shaped paper. Gold, for example, has been separated from platinum metals with ethyl acetate as solvent, and uranium from impurities. Conversely, bulk amounts can be freed from traces of impurities. Where, however, the trace of metal is contained in a large volume of solution, then some preliminary concentration of the trace may be necessary. Apart from the methods of concentration already indicated in previous sections, two other procedures are available, namely liquid–liquid extraction and co-precipitation. When the trace metals have been sufficiently concentrated, paper chromatography can be applied for further separation of the constituents. This may well have applications for dilute biological fluids, or natural waters such as sea-water.

The choice of filter-paper (or cellulose powder) has to be considered from the points of view of (i) mineral content,

FIG. 1. DISK CHROMATOGRAM



From Pollard & McOmie (1953) by permission of Butterworths Scientific Publications

Spot of aqueous solution of metallic nitrates placed at centre of paper (Whatman No. 1 or 3 MM); wick Y from paper dipped into solvent mixture consisting of acetone containing 5% water and 8% (v/v) concentrated HCl; run for about 2 hr.; dried and sprayed with rubeanic acid. Ni, blue; Co, brown; Cu, olive green; Fe, brownish green

For details, refer to Table I (i).

e.g., sodium, calcium, etc., (ii) strength of paper when wet, (iii) rate of flow, and (iv) thickness of paper. Defects in separations due to (i) may be corrected by using acid-washed paper. In quantitative estimations, corrections for cations present in the paper are made, e.g., in the determination of calcium, the amount of calcium in the paper may be of the same order as that separated on the chromatogram. In addition to washing with acid, the paper may need further treatment with (i) ethylenediaminetetraacetic acid to remove alkaline earths, (ii) 8-hydroxyquinoline for heavy metals, or (iii) hydrogen sulphide for fixing lead. Apart from these considerations, Whatman No. 1 filter-paper and ashless cellulose powder are the most used materials, and for many purposes these do not need any preliminary treatment before use in chromatography.

Many reagents are suitable for the location of the inorganic cations and anions after separation on paper or in the eluates and, since different colours for different ions can be obtained, the identification is particularly easy for many of the cations. Where this is so, the R_F values need not be absolutely reproducible, although sometimes, as with the rare earths, there is no specific test for each individual metal and then the R_F values must be known. The fluorescence effects under ultraviolet light with reagents like 8-hydroxyquinoline are invaluable, and it is sometimes advantageous to view coloured zones under ultra-violet light. Sensitive tests should be employed, and different reagents may vary quite considerably in this respect. The most sensitive of all tests are those with

radioactive tracers, when quantities as small as $10^{-6}\gamma$ have been detected.

The choice of solvent mixture for the mobile phase is made on the assumption that partition is the main process operating. Some metal salts are exceptionally soluble in organic solvents, and this, as for uranyl nitrate with ethyl ether, enables the salt to move to the solvent front and thus be completely separated from less soluble materials. The aim generally, however, is to use inorganic compounds which are only partially soluble in the organic phase, so that as the solvent moves over the paper, redistribution will occur many times between the two phases (the mobile phase, and the water held on the cellulose possibly as a water–cellulose complex). With even slightly different distribution coefficients, the repeated partition will eventually bring about a distinct separation of compounds into zones at different positions between the starting line and the solvent front.

Apart from the solvating effect of alcohols and ethers on inorganic compounds, there are three other ways by which a salt which is sparingly soluble in an organic solvent can be made to dissolve. These are based on reaction with the solvent itself or reaction with some reagent dissolved in the organic solvent. In the former category, one would place acetylacetone, collidine, and pyridine, all of which will form complexes with a metal ion, resulting in solution in these organic materials used as the mobile phase. For example, any copper salt will form a soluble collidine complex which moves with the collidine to the solvent front. In the second category are (i) the addition to the solvent mixture of the acid corresponding to the anion in the original salt, and (ii) the addition of a complex-forming reagent which will convert the original salt into a complex of the metal ion. An example of (i) is the addition of concentrated hydrochloric acid to the mobile phase so that normal chlorides can be converted into soluble complex chlorides. There are many examples of (ii) and the use of 8-hydroxyquinoline in *n*-butanol for the separation of the rare earths might be mentioned here. The formation of chelate complexes will depend on the equilibrium:

$$\text{metal ion} + \text{complexing agent} = \text{metal complex} + \text{hydrogen ion};$$

consequently the formation and subsequent existence of the complex will be intimately related to the pH of the mobile phase and the stability of the complex. If insufficient complexing reagent is included in the mobile phase, then all the initial material may not be converted into the complex, and so there would be tailing or the formation of two zones. Double zoning can also occur when there is competition for the metal ion between the original anion and either the added different anion, or the complex-forming reagent. If strontium chloride is eluted with a solvent mixture consisting of butanol containing a high concentration of acetic acid, two zones are formed on the chromatogram: the first (at R_F 0.1) is due to strontium chloride, while the second (at R_F 0.5) is strontium acetate. The latter zone trails backwards with decreasing intensity to the chloride zone.

One aspect of acidity has already been mentioned in connexion with complex-formation; another is the prevention of hydrolysis of the original salt. The presence of an acid is generally necessary in all solvent mixtures if separations of cations are to be satisfactory. For anions, the solvent mixture is usually alkaline, though for the phosphate separations acid solvents have been used as well as alkaline ones.

One of the most interesting roles that an acid can play is its effect on the affinity of cellulose for water through the adsorption of the strongly polar acid into the cellulose, and this will be discussed in relation to the general effect of water.

For some separations the water content of the solvent mixture must be kept low, but for others it is essential to keep the content high. Difficulties most often occur with solvents like acetone which are miscible with water in all proportions. It is found that whereas absolute methanol will move alkali metals to reasonable R_F values (K, 0.2; Na, 0.43; Li, 0.73), neither ethanol nor acetone will move them at all unless about 20% of water is present and then the R_F values with the ethanol solvent are K, 0.24; Na, 0.35; Li, 0.59.

With some solvent mixtures containing water, two solvent fronts are observed; the area between the starting line and the first front is wet as a result of absorption of water from the solvent by the cellulose, and the area between the two fronts is water-free. The relative position of the wet and the water-free solvent fronts depends not only upon the water content of the solvent mixture, but also upon its acidity. If a strongly polar acid is used, then the area of the water-free patch decreases with increasing acidity owing to the effect of the acid upon the absorbing property of the cellulose. On the other hand, with a weak acid like acetic acid, two fronts may not be observed. The importance of these two fronts lies in the fact that some cations move in the water-free area, and consequently the relative movement of these cations can be altered by shifting the positions of the two fronts.

Closely connected with the water content of the solvent is the humidity of the space within the tank. This is normally controlled by equilibration, but sometimes humidity is controlled more accurately by introducing saturated solutions of salts into the tank. The drying of the original salt spot after it has been placed upon the paper is important when work is being done with some solvent mixtures, e.g., acetone or ether.

To add to the complexity of the whole process, the following factors have to be considered: interference of one cation with others present in the original mixture, effect of anions, time of running of the chromatogram, temperature, and the nature of the cellulose support. Thus, a procedure which in fact is very simple in practice once the right conditions have been ascertained is full of complexities when one analyses theoretically the various factors involved.

By the use of a great variety of solvent mixtures, many groups of cations and anions have been separated; selected examples are listed in Table I, and illustrated in figs. 1 and 2.

5. Schemes of Analysis

Considerable progress has also been made in working out schemes of analysis which can be applied to unknown mixtures of cations. Two schemes are possible. One is based on the comparison of chromatograms prepared with different solvent mixtures, and the other on the systematic application of a differential spraying method to a number of chromatograms prepared with the same solvent mixture. The cations are identified partly by their R_F values and partly by characteristic chemical tests.

Where only a limited quantity of material is available for analysis, improved separations are obtained by a two-way process. The chromatogram is first run in one direction with a solvent and then in a direction at right angles by a second different solvent. Ebel has performed two-way

TABLE I. CHROMATOGRAPHIC SEPARATIONS OF CATIONS AND ANIONS

Separation	Solvent system				Salts used	Remarks on detection, etc.
	Organic solvent	Acid	Water (ml.)	Additional components		
(i) Ni, Co, Cu, Fe^{3+}	Acetone (87 ml.)	Conc. HCl (8 ml.)	5	—	Nitrates	Dried and sprayed with rubeanic acid. Ni, blue; Co, brown; Cu, olive green; Fe, brownish green
(ii) K, Na, NH_4, Li*	Methanol (70 ml.) *n*-Butanol (30 ml.)	—	—	—	Chlorides	Strips washed in silver nitrate to precipitate AgCl, then in water to remove excess $AgNO_3$, finally in ammonium sulphide, giving brown Ag_2S spots
(iii) Ba (0.25), Sr (0.45)†	Methanol (50 ml.) *iso*Propanol (30 ml.)	Formic acid (2 ml.)	15	Ammonium formate (2.5 g.)	Formates	(iii) Sprayed with sodium rhodizonate, giving red spots for Ba, Sr
(iv) Ba (0.25), Sr (0.45), Ca (0.60), Mg (0.75)						(iv) Sprayed with 8-hydroxyquinoline held over ammonia under ultra-violet light, giving green fluorescent spots
(v) U^{4+} (0.05), UO_2^{2+} (0.55), Fe^{2+} (0.35), Fe^{3+} (0.70)	Ether (50 ml.) Methanol (30 ml.)	Conc. HCl (4 ml.)	15	—	Chlorides	Held over ammonia to neutralize the paper. Sprayed with $K_3Fe(CN)_6$, giving U^{4+}, brown; Fe^{2+}, blue; Fe^{3+}, green. Sprayed with 8-hydroxyquinoline, viewed over ammonia under ultra-violet light, giving U^{4+}, UO_2^{2+}, brown; Fe spots, black
(vi) F′, Cl′, Br′, I′	Butanol (40 ml.) Pyridine (20 ml.)	—	to saturation	Ammonia (10 ml., sp. gr. 0.880)	Potassium	Take upper layer of solvent mixture. Detect as under (ii)
(vii) S_2O_3'' (0.1), thionic acids (S_nO_6'', n = 3→6), S_3O_6'' (0.32), S_4O_6'' (0.45) $H_2S_4O_6$ (0.65), $H_2S_5O_6$ (0.74), $H_2S_6O_6$ (0.82)	*iso*Propanol (50 ml.) Acetone (20 ml.)	—	30	Potassium acetate (2 g.)	Potassium or as free acids	Sprayed with silver nitrate, then warmed to decompose silver salts to Ag_2S. Afterwards chromatogram washed with thiosulphate solution to remove excess Ag

Paper: Whatman No. 1, acid washed (1: 5 conc. HCl) except in (ii) where untreated Whatman No. 1 was used.
Separation (v) was prepared by upward chromatography, the others by downward chromatography.
*** Separation (ii) reported by Sakaguchi & Yasuda (1951).**
† Figures in parentheses give R_F values.

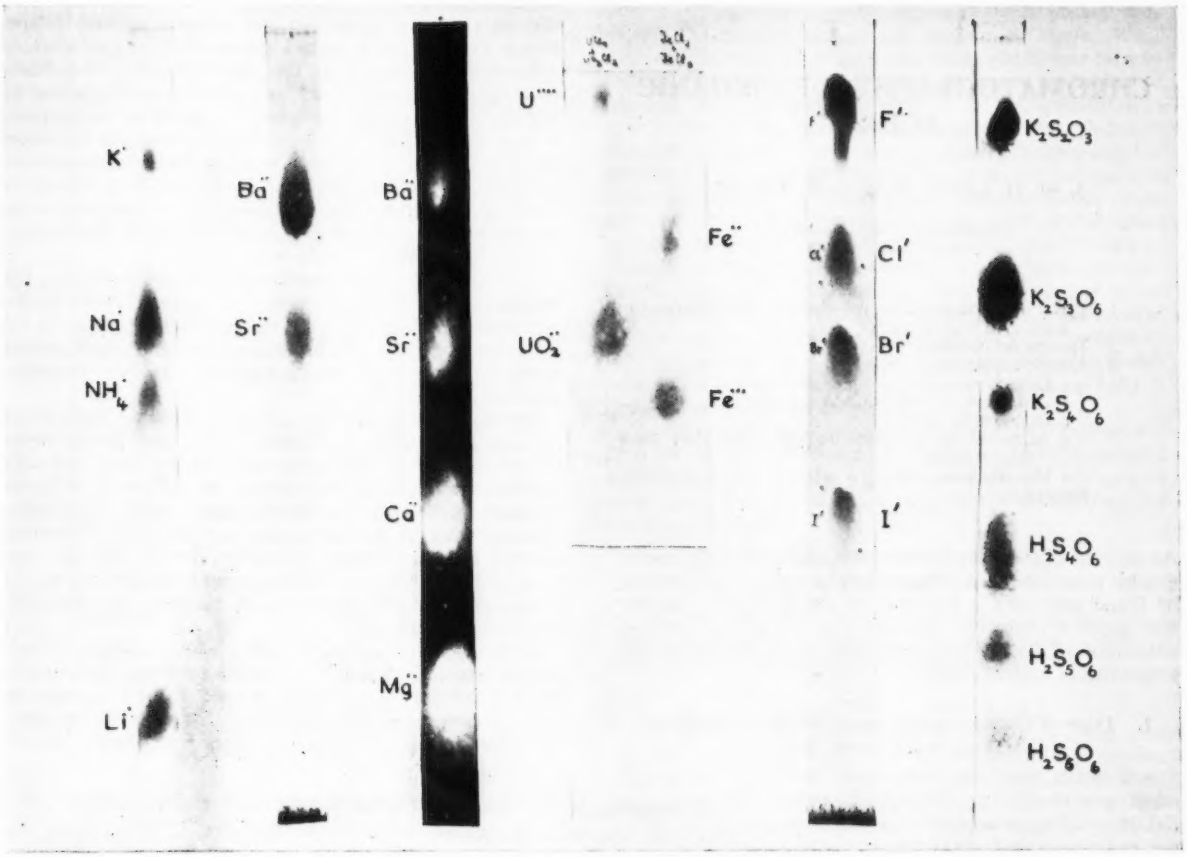
chromatography with hypophosphite, phosphite, orthophosphate, pyrophosphate and trimetaphosphate, by using a mixture of absolute alcohol and *iso*butanol with water and ammonia as the first solvent; and absolute alcohol with water, trichloroacetic acid and ammonia as the second solvent (Volmar, Ebel & Fawzi Bassili, 1953). It is also quite practicable to use two different solvents consecutively in the same direction; this may serve to separate ions which are insufficiently separated by the first solvent.

6. Quantitative Determinations

There are three main ways in which chromatography can be used for quantitative estimations, namely, (i) the separation of one particular ion from several others which may or may not be in excess of the one to be estimated, (ii) the separation of a group of ions which can then be individually estimated, and (iii) the separation of a mixture of ions into appropriate smaller groups such that the individuals in the smaller groups can be estimated by polarographic or spectrophotometric methods. The advantage of (iii) is that the final method of estimation may be made easier, since it would be arranged that the interfering ions would not be present together in the smaller group. On paper strips the estimation can be made for microgram quantities, while, if it is desired, cellulose columns can be employed for larger quantities.

Besides the methods of estimation which involve elution of the zone and the paper followed by some conventional analysis of the eluate, many attempts have been made to devise a direct determination of inorganic ions on filter-paper. The accuracy of such direct methods is generally not better than 5% for procedures which involve (i) measurement of the area of the spot with a planimeter, (ii) measurement of total colour density by scanning at constant intervals along the length of the chromatogram by a transmission densitometer, colori-

FIG. 2. SEPARATIONS OF CATIONS AND ANIONS



For details, refer to Table I (ii), (iii), (iv), (v), (vi) and (vii).

meter, or spectrophotometer, (iii) measurement of total colour density where the light transmitted through the entire spot is read with a densitometer or adapted absorptiometer, (iv) measurement of maximum colour density using absorptiometers or spectrophotometers after the paper has been made more transparent by treatment with a mixture of paraffin and bromonaphthalene, (v) radioactivity measurements made by scanning a chromatogram of radioactive elements. Vaeck (1954) claims an accuracy for nickel equal to that for the best colorimetric methods, by measuring the reflectance of the rubeanic derivative of nickel with a spectrophotometer. His method involves removing all interfering ions from the nickel by chromatography, and then estimating the nickel which is left on the starting line. Vaeck's method clearly has limitations; there is still no direct method which can generally be applied, and which gives an accuracy comparable with the frequently adopted elution method mentioned earlier. However, for many purposes such accuracy may not be required, and a direct method has much to commend it.

Interest in quantitative estimations has focused upon the determination of metals like uranium, gold, aluminium, titanium, iron, molybdenum, zinc, etc., and there is still need for more attention to elements which occur in the medical and biological fields. The determination of the alkali and alkaline earth metals in minerals has been studied by Seiler, Sorkin & Erlenmeyer (1952), while J. V. Martin, in this laboratory, is studying accurate methods for the alkaline earths. Some preliminary work on the estimation of arsenic has also been done.

The combination of chromatography with electromigration can extend the scope of the possible separations, and it is also possible to use the combined processes (electrochromatography) for the continuous separation of mixtures of cations—for example, silver and nickel.

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Continued at foot of page 192

CHROMATOGRAPHY OF ORGANIC ACIDS

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1 Types of chromatograms most commonly employed in work with organic acids
2 Theoretical considerations
3 Specific applications
a Lower fatty acids
b Fatty acids of intermediate chain length
c Higher fatty acids
d Acids of the tricarboxylic acid cycle, and some related acids
e Miscellaneous acids
References

An account of the more fundamental and derived chromatographic procedures and of the principles underlying them will be found elsewhere in this issue of the Bulletin.[1] The present paper is concerned basically with the application and adaptation of the different procedures to the separation and estimation of organic acids.

1. Types of Chromatograms most Commonly Employed in Work with Organic Acids

Adsorption was the mechanism first relied upon to effect the necessary differential distribution of organic acids between the phase-pairs but, following the introduction of partition for this general purpose by Martin & Synge (1941), attention has been devoted very largely to partition between two liquid phases as a means of effecting the differential distribution of organic acids. Again, in the more recent of those organic acid studies which have depended upon adsorption, frontal and displacement analytical methods have been employed rather than elution ones, whereas elution analysis has been used almost to the exclusion of other methods throughout the work done with partition chromatograms. In comparison with adsorption and partition, ion exchange appears to have evoked little interest as yet as a means of effecting satisfactory differential distribution of the organic acids. Co-ordinate bonds (Robinson, 1947) and homopolar bonds (suggested by Martin, 1949) have not, as yet, been employed for this purpose. It must be realized that it is unusual, however, to encounter instances of chromatography in which both the mechanism of solute distribution between phases and the form of analysis employed are of pure type. Often there is inadvertent but inevitable mixing of types (e.g., see Lugg, 1952). The position is shown schematically in Table I.

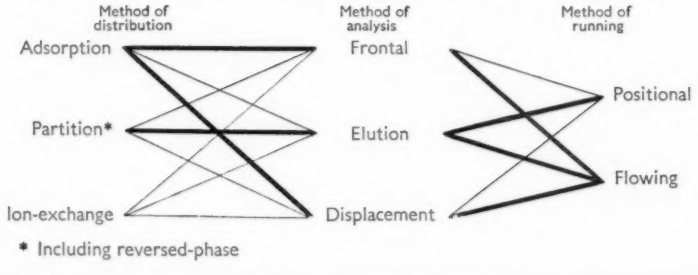
The forces which are usually predominant in holding the solutes in the stationary phase, viz. van der Waals in the adsorption, hydrogen bond in the partition, and ionic in the ion-exchange types of chromatograms, may be modified deliberately to achieve specific purposes. Relevant examples are discussed in section 2.

Partition chromatography on sheet supports, introduced by Consden, Gordon & Martin (1944), may be employed instead of partition chromatography on columns, and with considerable advantage in certain types of work. Whereas in column work with organic acids (and, indeed, with most solutes of late) the chromatogram is normally run as a flowing one (the solutes emerging in solution serially from the base of the column), the sheet chromatograms are normally run in positional style (the solute bands assuming characteristic positions on the sheets).

Reversed-phase chromatography, which employs a supporting material selected to retain the less polar phase and in this way reverses the conventional roles of the two phases in partition chromatography, has recently found interesting applications in both column and sheet work with organic acids.

TABLE I. SCHEME OF CHROMATOGRAPHIC PROCEDURES

Connecting lines show the possible combinations, the heavier lines indicating the commoner combinations in use with organic acids. In current practice, column chromatograms are almost invariably run in flowing, and sheet chromatograms in positional, style.



* Including reversed-phase

[1] See Williams, p. 165 of this number of the Bulletin. —ED.

INORGANIC CHROMATOGRAPHY

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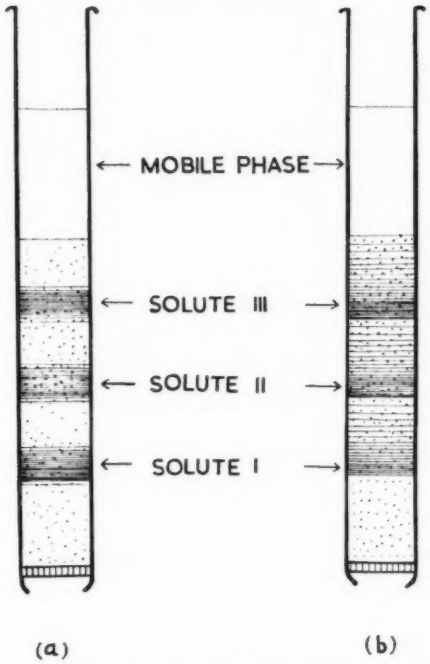
FIG. 1. COLUMN CHROMATOGRAMS OF A MIXTURE OF THREE ORGANIC ACIDS, WITH ELUTION ANALYSIS



a: showing suppression of tailing of the bands
b: showing tailing of the bands

2. Theoretical Considerations

The more fundamental theoretical treatments (e.g., those of Wilson, 1940; Martin & Synge, 1941; Mayer & Tompkins, 1947) need not be the close concern of this contribution, as they are dealt with elsewhere.[2] Here it is necessary only to consider aspects which are of special relevance to the separation of organic acids.

With distribution isotherms which approximate to linearity, such as characterize the partitioning of many simple solutes between two liquids, the bands are ordinarily symmetrical and compact (see fig. 1a). But an organic acid (*HA*) is represented by *HA* molecules and H^+ and A^- ions in each phase. As the degree of ionization and the solubility of an ion will ordinarily be much higher in the more polar than in the less polar phase, the gross partition coefficient can be expected to vary with concentration. Consequently, when the more polar phase is stationary (the usual condition), tailing of the solute band will occur similar to that customarily seen in adsorption chromatography (fig. 1b). A similar effect is encountered if the un-ionized molecules can associate readily, especially in the mobile phase, and can be avoided only by sufficiently radical change in the mobile phase solvent.

Lester Smith (1942) discussed the ionization problem but offered no solution. It has since been solved in a variety of ways: Isherwood (1946) suppressed ionization of the solute in the more polar phase by acidifying it with a foreign mineral acid, Lugg & Overell (1947) swamped both phases with volatile foreign acid, Scarisbrick, Baldwin & Moyle (1948) used columns in which the more polar phase was heavily buffered, under which condition the degree of ionization of the acid was no longer determined by its concentration therein but by the pH of that phase, and was therefore relatively constant. This last method also overcame another difficulty. The higher fatty acids are of such low solubility in water that they tend to travel together with the mobile phase front: their *R* values (Martin & Synge, 1941) or R_F values (Consden *et al.* 1944) are too great. If, however, the pH of the stationary phase is maintained at a value such that substantial (constant) fractions of the acids are present in the form of ions (A_1^-, A_2^-, etc.) and relatively little in the form of molecules (HA_1, HA_2, etc.), the gross partition coefficients may be such as to permit excellent resolution. It is this problem which reversed-phase partition chromatography is designed to solve otherwise.

Martin (1949) has provided a theory of displacement analysis in its application to partition chromatography, dealing more specifically with the resolution of mixtures of weak acids in chromatograms with alkali- or buffer-loaded stationary phases.

3. Specific Applications

The examples considered in this section, representing a small fraction of all publications, have been selected primarily to illustrate problems and their solutions.

a. Lower Fatty Acids

As these acids are rather volatile in the un-ionized form care must be exercised to avoid losses by vaporization. Column methods will afford protection, sheet methods will not, and the latter must be reserved for modifications in which the acids are present largely in the form of their anions or are otherwise rendered non-volatile.

The differences between adsorption or partition coefficients of the free acids would suggest that their mixtures should be resolvable by elution analysis in adsorption or partition chromatography, despite the tailing. Actually, Nestler & Cassidy (1950) were disappointed in the behaviour of the acids on charcoal adsorption columns in elution analysis. By frontal analysis they obtained sharp fronts with mixtures of acetic, propionic and butyric acids but were unable to make satisfactory quantitative estimations. However, Holman & Hagdahl (1948, 1950), in displacement analysis studies embracing the fatty acids from C_1 to C_{22}, were able to resolve mixtures by using picric acid or higher fatty acids as displacing agents. Thus, the C_6 acid was used to effect a separation of acids ranging from C_1 to C_5.

Greatest success has attended the use of partition methods. Lester Smith (1942) separated and estimated formic, acetic, propionic, butyric and valerianic acids by elution analysis on a moist silica-gel column containing an acid-base indicator to reveal the positions of the acid bands. Run off, in flowing style, the separate acids were then titrated. The method was developed by Ramsey & Patterson (1945) and by Elsden (1946), who modified the mobile phase composition and made other alterations, and by Peterson & Johnson (1948), who replaced the silica gel with Celite 545 and used a series of mobile phases. Although the bands are advantageously revealed by the

[2] See Williams, p. 165 of this number of the Bulletin.—Ed.

internal indicator, these procedures suffer from the disadvantage of tailing.

For sheet chromatography the conversion of the acids into the non-volatile hydroxamate derivatives by Fink & Fink (1949) seems less acceptable than working with alkaline phases. Brown & Hall (1950), Hiscox & Berridge (1950), Long, Quayle & Stedman (1951) and Kennedy & Barker (1951) are among those who have explored these possibilities. In recent extensions of these studies, Reid & Lederer (1952) and Isherwood & Hanes (1953) have separated the acids on paper, ostensibly with a single phase comprising an organic solvent mixed with aqueous ammonia, but in effect there may be two liquid phases (see Synge, 1949). Both these phases would be strongly polar, holding the acids in approximately constant fractions, and almost exclusively as anions. These workers have adapted their techniques to quantitative use.

b. Fatty Acids of Intermediate Chain Length

The views of Martin (1949) would suggest that for any particular pair of immiscible solvents the partition coefficient would change by a definite factor for each step along an homologous series. But the factor diminished markedly above the C_5 acid with the saturated fatty acids (Isherwood & Hanes, 1953). Thus, in the partition chromatography of intermediate members, the differences in R or R_F values being small, appreciable tailing cannot be tolerated. Again, the solubilities of some of the acids in water are inconveniently small.

Ramsey & Patterson (1948a) achieved satisfactory resolution of C_5–C_{10} acids by the use of methanol and *iso*-octane as stationary and mobile phase solvents, respectively, with bromocresol green indicating band positions in the columns during elution analysis. The degrees of ionization in the methanol were sufficient to affect the indicator but not such as to cause appreciable tailing. Nijkamp (1951) has adapted the method to semi-microscale operation. A different approach was made by Scarisbrick *et al.* (1948) and Moyle, Baldwin & Scarisbrick (1948) who, employing moist silica-gel columns of pH values so well stabilized by buffers or otherwise that tailing was negligible, effected excellent resolutions by flowing elution analysis. The great disadvantage of this method is that the chromatogram must be run "blind". The extent of the bands was determined by serial withdrawal and titration of eluate.

These two methods appear to be superior in resolving power to that of Peterson & Johnson (1948), who used, as stationary phases, 28–36 N solutions of sulphuric acid supported on Celite 545, and mobile phases prepared from benzene.

In their work on the C_2–C_8 acids, Fairbairn & Harpur (1951) took no special steps to control ionization, and the tailing which troubled them may have been due more to this fact than (as they seem to have believed) to adsorption by the supporting material.

c. Higher Fatty Acids

The long chain length and consequent great importance of van der Waals forces would suggest adsorption chromatography as the method of choice for fine resolution of mixtures in this group, except in elution analyses where tailing might be serious. However, technical difficulties, such as a tendency towards irreversible adsorption, obtrude.

In partition chromatography the low solubilities of these acids in non-alkaline aqueous media tend to impose restrictions, but by reversed-phase modifications the R or R_F values may be brought within the useful ranges. In any event, the differences between the R or R_F values (like those between the partition coefficients) tend to be small and it is consequently important to minimize tailing.

Using adsorption columns, earlier workers (Kondo, 1937; Kaufmann, 1939) effected partial resolution of fatty-acid mixtures derived from oils. In a more successful study di Modica & Rossi (1952), using elution analysis with acetone as mobile phase, were able to resolve mixtures of oleic, linoleic and linolenic acids at low temperature on alumina adsorption columns. Claesson (1946) successfully employed adsorption frontal analysis with acids of this group but found displacement analysis unsatisfactory. More recently Holman & Hagdahl (1948, 1950) have had considerable success in the adsorption displacement analysis of these acids.

Ramsey & Patterson (1948b), extending their studies to the higher fatty acids (C_{11}–C_{19}), obtained good resolutions with a stationary phase prepared from equal weights of furfural alcohol and 2-aminopyridine supported on silica gel and with hexane as mobile phase solvent in flowing elution analysis, run "blind" of necessity. The large amount of weak base would have the effect of buffering the stationary phase—even so, only mixtures of alternate members of the homologous series could be resolved satisfactorily.

Following earlier reversed-phase chromatography experiments by Boscott (1947), Boldingh (1948) developed a technique for the resolution of mixtures of long-chain fatty acids as their methyl esters. Subsequently, Boldingh (1949) announced the successful resolution of mixtures of even-numbered carbon-atom acids (C_6–C_{24}) on partition columns, using powdered rubber, swollen with benzene, as stationary phase, and water–methanol–acetone mixtures as mobile phases.

Howard & Martin (1950), for the resolution of mixtures of the C_{12}–C_{18} fatty acids by flowing elution analysis, have employed kieselguhr treated with dichlorodimethylsilane as support for *cyclo*hexane, *n*-octane or medicinal paraffin as stationary phase, the mobile phase being aqueous methanol for the first two and aqueous acetone for the last one. They observed that the bands, as reflected in the titration curves, were more widely spread the larger the proportion of water in the mobile phase. This effect and the evident asymmetry of some of the titration curves (such bands travel tail first in reversed-phase work) could well be due to appreciable ionization in somewhat polar mobile phases.

d. Acids of the Tricarboxylic Acid Cycle, and Some Related Acids

Many of these acids were becoming of great interest in metabolic studies at about the time of the revival of interest in chromatography. They have not, generally, been found readily amenable to adsorption chromatography and their routine resolution had to await the development of the partition chromatographic methods.

These acids are mostly stronger than those that have been dealt with so far, and the problem of ionization in their partition chromatography is correspondingly more serious: on the other hand they can be chromatographed on sheets as the free acids because their volatilities are negligibly low.

By flowing elution analysis in partition columns, Isherwood (1946) was able to resolve quantitatively mixtures of fumaric,

succinic, oxalic, malic, citric and tartaric acids. The stationary phase solvent was 0.5 N-sulphuric acid solution (to suppress ionization) supported on silica gel. The mobile phase solvents were *n*-butanol–chloroform mixtures. Dropwise addition of indicator solution to the issuing percolate permitted recognition and separation of the bands.

Lugg & Overell (1947, 1948) resolved mixtures of these acids by one-dimensional and two-dimensional sheet partition chromatography. In the presence of the swamping acid (particularly formic acid), test acids were partly lost by esterification if alcohols were allowed to enter into the composition of the phases. In development of these studies (Bryant & Overell, 1951, 1953; Overell, 1952), ion-exchange chromatography has been used to separate the plant acids as a group from contaminants, and measurement of " comet " lengths with unacidified phase systems, and of band areas with acidified phase systems, have been used to estimate the individual acids.

Isherwood & Hanes (1953) have also provided a means of estimating some of the acids of this group after resolving the mixtures by the method referred to in section 3a.

e. Miscellaneous Acids

To give perspective, reference must be made finally to certain examples not otherwise dealt with in previous sections.

Dicarboxylic acid mixtures have been subjected successfully to adsorption frontal analysis and estimation by Claesson (1946), and separations adequate for purposes of estimation have been effected on paper-sheet chromatograms by Isherwood & Hanes (1953). It is of interest to note, in this connexion, that higher members of the homologous dicarboxylic acid series accord better than do the corresponding monocarboxylic acids with the postulate of Martin (1949; and see section 3b of this paper).

Hydroxy- and keto-monocarboxylic acid mixtures have been resolved by Liberman, Zaffaroni & Stotz (1951) on paper-sheet partition chromatograms, with the aid of acidified phases. The handicap of low R_F values was overcome by allowing the solvent front to overrun the sheet.

The paper-sheet partition chromatography of aromatic acids has been studied by several investigators, including Evans, Parr & Evans (1949), Bray, Thorpe & White (1950) and Fewster & Hall (1951), all of whom, in various ways, stabilized the pH values of the phases.

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CHROMATOGRAPHY OF PEPTIDES

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1 Partition chromatography
  a Paper chromatography of peptides
  b Detection of peptides on paper
  c Application of paper chromatography
  d Chromatography of peptides on starch
  e Partition chromatography of peptide derivatives
2 Adsorption chromatography of peptides and coloured derivatives
  a Adsorption on silica
  b Adsorption on charcoal: displacement chromatography
  c Counter-current methods
3 Ion-exchange chromatography
  a Displacement chromatography on ion-exchange resins
  b Elution development of ion-exchange resin columns
4 Conclusion
References

For the purpose of this review a peptide is defined as a substance formed from amino acids linked together in amide linkage and with a molecular weight below 10,000. The limit of 10,000 is arbitrary but it has some physical significance since below this molecular weight polypeptides are not subject to heat denaturation whereas when the molecular weight rises much above this figure heat denaturation is of vital importance. The loss of biological activity on denaturation suggests that, above a certain molecular size, activity is governed not entirely by amino-acid sequence in the peptide chain but also by the way in which the chain is folded and held together.

The development of chromatographic methods for the separation of peptides has had two main objectives, firstly the purification of naturally occurring substances such as the pituitary hormones or the antibacterial polypeptides and secondly the isolation of smaller peptide fragments present in partial hydrolysates of these substances and of proteins. Although it is impossible to divide chromatographic methods into rigid categories, it was convenient for the purpose of this review to divide the available methods into three types: partition chromatography, adsorption chromatography, and ion-exchange chromatography. The literature on the subject is extensive and we have made no attempt to be comprehensive but instead have tried to emphasize those methods which we believe to be generally applicable. More extensive reviews, by Synge (1949a), Sanger (1952) and Lederer & Lederer (1953), are available.

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1. Partition Chromatography

a. Paper Chromatography of Peptides

Peptides may be chromatographed on paper by the techniques developed for amino acids. Several lists of R_F values have been published (Consden, Gordon & Martin, 1947; Cook & Levy, 1950; Pardee, 1951; Heyns & Anders, 1951; Knight, 1951; Grassmann & Deffner, 1953). In general, the solvents used for amino-acid chromatography are suitable for small peptides, but many peptides have rather high R_F values in phenol; *m*-cresol retains the advantage of phenol as a proton donor (Martin, 1949) and gives a lower range of R_F values (Consden, Gordon & Martin, 1947).

It is possible, from a knowledge of the amino-acid composition of a small peptide, to make an estimate of its probable R_F in any solvent system. Martin (1949) suggested on theoretical grounds that the partition coefficient of a peptide divided by the product of the partition coefficients of the constituent amino acids in the same solvent system would be a constant; thus, $\alpha_{AB}/\alpha_A\alpha_B = K$, where α_{AB}, α_A and α_B are the partition coefficients of the peptide and of the individual amino acids respectively. The R_F value depends on partition coefficient so that the R_F value of a peptide can be calculated in terms of the R_F values of the constituent amino acids and a constant, K, which can be determined experimentally. The method has been found useful in checking the identity of unknown peptides (Sanger, 1952).

Although paper chromatography has been used mainly for the separation of small peptides the method is applicable to some large peptides. Several of the recently isolated antibiotics have been found to be peptides. The polymyxins were first separated by paper chromatography using butanol–acetic acid as solvent (Jones, 1948), and three licheniformins with molecular weights approaching 5,000 were sharply separated from one another in a mixture of collidine, lutidine and ammonia (Callow & Work, 1952). The use of paper is limited, however, by the tendency of many larger peptides to become irreversibly adsorbed on the paper with resultant "tailing". For the separation of large peptides, partition on kieselguhr (Porter, this number of the Bulletin, p. 237), counter-current distribution (see section 2c), or chromatography on ion-exchange resins (see section 3)[1], is likely to give better results.

b. Detection of Peptides on Paper

Some peptides, particularly those containing lysine residues, can be detected on paper after chromatography by spraying with the 0.1% ninhydrin reagent normally used for amino acids, but with larger neutral or acidic peptides the colour may be so weak as to be missed entirely and much more satisfactory results can be obtained with the ninhydrin reagent suggested by Lewis (1952; cf. Agren & Glomset, 1953). This contains 0.4% ninhydrin in a mixture of water, *iso*propanol and collidine (10:90:5). However, even this reagent fails to detect cyclic peptides or diketopiperazines and Rydon & Smith (1952) suggested that the paper should be exposed to gaseous chlorine, which reacts with the amide linkage of peptides to form an unstable chloroimide. The chloroimide itself is colourless but if the paper is then sprayed with a solution of starch and potassium iodide an intense blue spot is produced by decomposition of the chloroimide and liberation of iodine. In practice the method gives rather variable results but the modification suggested by Reindel & Hoppe (1953), in which

[1] See also Partridge, p. 241 of this number of the Bulletin.—Ed.

the paper is treated with chlorine dioxide followed by benzidine, has proved quite satisfactory (Elliott & Hooper, personal communication).

Where paper chromatography is being used to isolate small quantities of peptides it may be impractical to use one chromatogram sprayed with colour reagent as a guide to the positions of peptides on another sheet. In this case the peptide may sometimes be detected by heating the paper at 100° C. for about 20 minutes and then examining for fluorescence under ultra-violet light (Phillips, 1948), but it is then essential to avoid the presence of any phenol on the paper. Alternatively the method of Boissonnas (1950) may be used, in which the paper is covered with a series of tiny spots of ninhydrin reagent or the paper may be sprayed with 0.025% ninhydrin (Sanger & Tuppy, 1951a); this causes little destruction of peptide but of course fails to show the presence of the less chromogenic substances.

c. Application of Paper Chromatography

The value of paper chromatography in determining the structure of a peptide was clearly demonstrated by the studies of Consden, Gordon, Martin & Synge (1947) on gramicidin S. More recently Sanger & Tuppy (1951a, 1951b) and Sanger & Thompson (1953a, 1953b) have shown that even rather complex peptide mixtures may be resolved by two-dimensional chromatography on paper, and, largely using this method, they have been able to isolate a sufficient number of peptides from partial hydrolysates of insulin to permit determination of the complete amino-acid sequence of this protein. For this type of fractionation a rather thick paper (Whatman No. 3) is convenient as larger quantities of peptides can then be fractionated.

When paper is used to isolate peptides particular care must be exercised that the results are not vitiated by the presence of extractable peptides in the paper. A suitable apparatus for washing large sheets before use has been described by Isherwood & Hanes (1953). The same difficulty of contamination may arise when paper pulp or starch columns are used to separate peptides.

d. Chromatography of Peptides on Starch

Chromatography on columns of potato starch, which was originally described by Elsden & Synge (1944), has been developed into an accurate method for the quantitative determination of mixtures of amino acids by Stein & Moore (1948) and Moore & Stein (1948a, 1949). This method is also capable of effecting the separation of peptides. Thus Moore & Stein (1948b) showed that it was possible to separate leucylglycine from glycylleucine on a starch column. Ottesen & Villee (1951) have separated the peptides produced on the conversion of ovalbumin to plakalbumin by means of starch columns. Synge (1949b) fractionated a partial hydrolysate on starch and was able to isolate a number of di- and tri-peptides but, like Stein & Moore (1949), came to the conclusion that the method was not suitable for larger peptides. This has recently been confirmed by Fels & Tiselius (1952), who showed that the large peptide isolated by Borsook, Deasy, Haagen-Smit, Keighley & Lowy (1949) from liver by starch chromatography consisted of at least five components.

e. Partition Chromatography of Peptide Derivatives

N-Acetyl peptides like *N*-acetylamino acids can be separated by partition on silica gel columns (Gordon, Martin & Synge, 1943) but the acetylpeptides are colourless and it is more convenient to fractionate a coloured mixture of dinitrophenyl-(DNP-) peptides (Sanger, 1949). In the preparation of DNP-peptides and amino acids, good yields are obtained by avoiding excess of bicarbonate or fluorodinitrobenzene (Schroeder & LeGette, 1953). Alternatively a volatile base may be used to control the pH during reaction between peptide and fluorodinitrobenzene and then removed in vacuo (Sanger & Thompson, 1953a).

The preparation of a suitable silica is important and is not always achieved. The variables involved in the preparation are not fully controllable and despite careful adherence to published descriptions the product is sometimes useless (Tristram, 1946). In our experience the method of Porter (1950) usually gives good results but no two batches of silica have the same properties. Occasionally a batch which was found to be useless immediately after preparation has become quite satisfactory after six months. The variable behaviour of silica gel can be reduced by wetting with aqueous buffer instead of water (Blackburn, 1949; Middlebrook, 1951) but in general a silica which gives poor separations when unbuffered also gives poor separations when buffered. There is often less spreading of bands on a buffered column than on an unbuffered one, but the main advantage of using buffer is in the resolution of rather complex peptide mixtures. With unbuffered columns it is difficult to separate a mixture containing more than about six peptides, but by using buffer first at pH 4, then at pH 5.5, and finally at pH 7, it is quite easy to separate 12 or more components from a mixture.

Columns can be made with kieselguhr instead of silica as the solid support for the aqueous phase (Perrone, 1951; Kroner, Tabroff & McGarr, 1953) but there is also variation in the quality of kieselguhr, and in our experience a good quality silica gives separations superior to those obtained on kieselguhr.

Columns are preferable to paper where more than 200 μg. of material have to be fractionated and also where isolation rather than identification of the peptide is desired but, when the qualitative analysis of a mixture is sufficient, partition on paper offers advantages. Various solvent systems have been suggested (Monier & Pénasse, 1950; Biserte & Osteux, 1951; Rovery & Fabre, 1953). Particularly compact spots are obtained by the use of buffered paper (Blackburn & Lowther, 1951; Felix & Krekels, 1952). The resolving power of a paper strip for DNP-peptide is lower than that of a good silica column but the technique is easier and the resolving power may be increased greatly by the use of two-dimensional chromatography on paper.

In fractionation of complex mixtures of peptide a stage may be reached where the product is thought to be pure. With a DNP-peptide this can be checked to some extent by hydrolysis and determination of the end groups (Sanger, 1949). If the peptide has been provisionally identified a particularly sensitive test is to convert to the ^{131}I-pipsyl (*p*-iodotoluenesulphonyl) peptide, to mix this with a synthetic sample of pipsylpeptide containing ^{35}S, instead of ^{131}I, as the radioactive element and to chromatograph the mixture on paper. If the two substances are identical there will be a uniform distribution of the ratio ^{35}S/^{131}I over the whole area of the spot (Velick & Udenfriend, 1953).

2. Adsorption Chromatography of Peptides and Coloured Derivatives

a. Adsorption on Silica

Although adsorption on the solid support influences the behaviour of peptides separated on wet silica or wet paper, partition is the dominant influence. Dry silica mixed with kieselguhr can also be used to fractionate DNP-peptide and in this case adsorption is dominant (Green & Kay, 1952; Schroeder & Honnen, 1953; Schroeder, Honnen & Green, 1953). In this method also, variability in the quality of silica influences the behaviour of columns. The technique should be regarded as complementary to partition on silica rather than as a rival method. The physical forces involved are different, and peptide mixtures can be separated into different groups by the two methods. The chief advantage of the adsorption column is that it can carry about four times the quantity of DNP-peptide which could be used on a similar partition column. Adsorption on talc has also proved useful for preliminary separation of DNP-peptides (Sanger, 1949).

When DNP-peptides are hydrolysed with concentrated HCl in order to determine the nature of the end group there is frequently considerable decomposition of the DNP-amino acid. This can be overcome to some extent by the use of perchlorate (Hanes, Hird & Isherwood, 1952). The difficulty can also be overcome by the use of 3:5-dinitro-4-dimethylaminophenyl *iso*thiocyanate which gives with peptides the brightly coloured dinitro-dimethylaminophenyl-thioureido-peptides. These are sufficiently stable to be separated by adsorption chromatography on alumina and have the advantage that the terminal amino acid can be readily split off as a 4-dimethylamino-3:5-dinitrophenyl-thiohydantoin without rupture of any except the *N*-terminal peptide linkage (Reith & Waldron, 1954). The method looks promising but has not yet been applied to the analysis of complex peptide mixtures.

b. Adsorption on Charcoal: Displacement Chromatography

If a mixture of dissolved substances is allowed to flow through a column of powdered charcoal and is followed by a substance with an adsorption affinity higher than that of any of the substances to be separated, a procession of adjoining zones of characteristic concentrations is set up, each zone representing a pure component. The chief advantage over the adsorption-elution technique is that it avoids tailing of the bands and uses to the full the adsorption capacity of the charcoal. The method has been developed mainly by Tiselius (1943). Theoretically, the efficiency of the method is high (Martin, 1949) but there is a disadvantage that there are no blank zones between different components of a mixture. This difficulty can be overcome to some extent by interposing between the zones a number of compounds of intermediate adsorption affinities which can be removed by distillation or extraction. The method has been successfully used to check the purity of antibiotic peptides in the molecular weight range 2,000–6,000 and has been applied to the separation of synthetic peptides (Synge & Tiselius, 1947, 1949). It is particularly useful in the fractionation of complex peptide mixtures as a means of removing peptides otherwise difficult to fractionate because of their high adsorption affinity (Askonas, Campbell & Work, 1954). The ion-exchange method of Partridge (see section 3)[2] is essentially an application of displacement chromatography, using ionic affinities rather than adsorption affinity.

[2] See also Partridge, p. 241 of this number of the Bulletin.—Ed.

c. Counter-Current Methods

It is sometimes difficult to find a suitable solid support for partition of the larger peptides owing to tailing, probably caused by adsorption on the solid support. This difficulty can be overcome by liquid–liquid counter-current distribution. This process is essentially an extension of the normal partition in a separating funnel between immiscible solvents. It has the advantage that large quantities of material may be fractionated and more particularly that in the fractionation of large peptides difficulties due to irreversible adsorption are eliminated. The method has been mainly developed by Craig (1950, 1952) and Craig, Hausmann, Ahrens & Harfenist (1951), who have designed an automatic machine for carrying out successive extractions and have purified insulin and various antibiotic peptides (Craig, Gregory & Barry, 1950; cf. Newton & Abraham, 1950). A simpler apparatus has been described (Lathe & Ruthven, 1951) and even standard laboratory separating funnels can be used (Callow & Work, 1952; cf. Bush & Densen, 1948). The chief disadvantage of the method is that it is laborious. To reach the theoretical efficiency of a conventional chromatographic method, many hundreds of successive extractions are required. On the other hand the behaviour of any one peptide in a mixture can be rather accurately predicted from a knowledge of its distribution between the two solvents used.

3. Ion-Exchange Chromatography

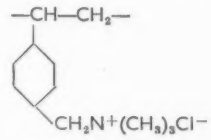
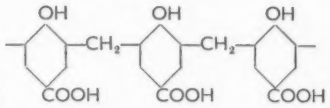
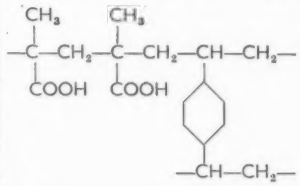
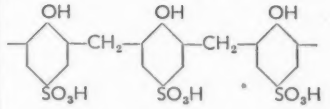
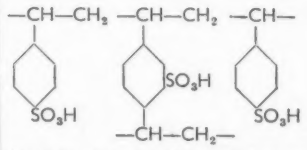
Since most peptides are ampholytes they may be fractionated by means of ion-exchange resins in the same way as amino acids. In addition to having ion-exchange properties, resins also have the property of binding substances by van der Waals forces. Thus aromatic amino acids are bound to resins more strongly than non-aromatic amino acids with similar ionic properties. In this respect peptides, especially large ones, may be expected to resemble more closely the aromatic amino acids than the neutral amino acids of low molecular weight.

Table I gives the names and some of the properties of the resins commonly used for separation of amino acids and peptides. Similar resins are on the same horizontal line and for most purposes these can be regarded as interchangeable.

a. Displacement Chromatography on Ion-Exchange Resins

We have already explained, in discussing the use of charcoal, the principles involved in displacement chromatography. Resin in a suitable column is first converted to the appropriate free acid or free base form. After addition of the peptide mixture displacement is achieved with an ion showing a higher affinity for the resin than any peptide. As with charcoal, a procession of adjoining zones is set up in the column and as development continues each zone is eventually displaced from the column by the substance following it. The method has the advantage that full use is made of the capacity of the resin, which may hold as much as one-third of its own weight of peptide, but there is serious overlap between successive zones. The method is of value mainly in large-scale work, and in our experience displacement methods do not give good results with less than about 5 g. of material. The main value of the method is probably in the separation of groups of similar peptides. Consden & Gordon (1950) have used the method to adsorb selectively the cysteic acid peptides from an oxidized partial hydrolysate of wool.

TABLE I. ION-EXCHANGE RESINS

Type	Functional groups	Manufacturer			Basic formula
		Permutit Co. Ltd. (Great Britain)	Rohm & Haas * (USA)	Dow Chemical Co. (USA)	
Weak anion	Primary, secondary or tertiary amine	De-Acidite E	Amberlite IR-4B Amberlite IR-45		Phenol-formaldehyde or polystyrene
Strong anion	Quaternary ammonium	De-Acidite F De-Acidite FF	Amberlite IR-410 Amberlite IRA-400	Dowex 2 Dowex 1	
Weak cation	Phenolic Carboxyl	Zeo-Karb 216			
	Carboxyl	Zeo-Karb 226	Amberlite IRC-50 (XE-64)		
Strong cation	Phenolic Sulphonic acid	Zeo-Karb 215	Amberlite IR-105 Amberlite IR-100		
	Sulphonic acid	Zeo-Karb 225	Amberlite IR-120	Dowex 50	 Contain approx. 12% divinylbenzene
		Zeo-Karb 225 (W.R. 1.1)	Amberlite IR-112	Dowex 50 × 4	Contain 4–6% divinylbenzene

* Distributed in Great Britain by British Drug Houses Ltd.

b. Elution Development of Ion-Exchange Resin Columns

In this method the resin is not used in its free acid or free base form but it is first equilibrated with a suitable buffer. The peptide mixture is added in the same buffer and a competition is set up between the buffer ions and the ionizable groups of the peptides for attachment to the active groups of the resin. As with other chromatographic methods, the forces involved are not entirely of one kind and in addition to ionic binding van der Waals forces probably play a significant role. The rate of development can be controlled by suitable variation of the ionic strength or of the pH of the buffer.

Elution development on Dowex 50 has been applied by Moore & Stein (1951) to the fractionation of amino-acid mixtures. Sodium citrate, sodium bicarbonate and sodium carbonate buffers were used over a pH range 3.4–11. The capacity of the resin is small (about one three-hundredth of the capacity in the displacement method) but the resolving power is such that all the amino acids present in a protein hydrolysate can be separated. Moore and Stein found that it was essential to use very fine bead resins (200–400 mesh/in.)[3] and low flow-rates.

The method of Moore and Stein has been applied to peptide mixtures obtained by partial hydrolysis of gelatin (Schroeder *et al.* 1953). Although some peptides were isolated from the effluent by conversion to their dinitrophenyl derivatives followed by adsorption chromatography on silica, it is not at all clear what degree of fractionation was actually achieved on the ion-exchange resin.

Effect of cross-linking. An ion-exchange resin may be prepared as a linear polymer, for example by polymerization and sulphonation of styrene, but such a resin has low mechanical

[3] 1 in. = 2.54 cm.—Ed.

stability, is of a rubbery consistency and undergoes great change in volume with change in pH or ionic strength of the medium. It is thus unsuitable for use in columns.

It will be noticed that several chemically similar resins have been produced with different percentages of cross-linking agent (divinylbenzene). This agent plays no part in ion exchange but binds the linear chains together and gives the resin greater physical stability. Too many cross-links, however, may prevent access of large molecules to the ion-exchange centres (Kunin, 1949; Richardson, 1949, 1951). Thus, while a closely cross-linked resin may be suitable for fractionation of amino acids, a resin with a lower percentage of cross-linking agent is preferable for the fractionation of peptides.

The usual form of Dowex 50 contains about 12% of divinylbenzene as cross-linking agent (Stein, 1953). Moore & Stein (private communication) have found that a similar resin with about 5% divinylbenzene gives much sharper fractionation of amino acids, and Dowmont & Fruton (1952), using this resin, have obtained excellent fractionation of some synthetic peptides. In this laboratory, we have experimented with a series of resins prepared by Dr K. W. Pepper at the Chemical Research Laboratory (National Physical Laboratory, Teddington, Middlesex) and by Dr T. R. E. Kressmann at Permutit Ltd. Sulphonated polystyrene resins with less than 2% divinylbenzene were difficult to handle because of large volume changes with alteration of pH.

For the fractionation of small peptides present in a partial hydrolysate of β-lactoglobulin, a resin with the cross-linkage recommended by Moore and Stein was found to give excellent results (Askonas, Campbell & Work, unpublished observations). For the fractionation of very large peptides, the polyacrylic acid resin Amberlite IRC-50 has been found particularly valuable. It can be used with ammonium or sodium buffers (Kroner *et al.* 1953; Richter, Ayer, Bazemore, Brink & Folkers, 1953) and has given good results in the purification of corticotrophin B, a peptide with a molecular weight in the 5,000 range. Proteins have also been fractionated on this resin (Paléus & Neilands, 1950; Dixon, Moore, Stack-Dunne & Young, 1952; Tallan & Stein, 1953; Hirs, Moore & Stein, 1953; Boardman & Partridge, 1953).

Gradient elution. Tiselius (1952) and Alm, Williams & Tiselius (1952) have described an important method of elution of chromatographic columns which they term gradient elution. In this method, the solvent used to develop the chromatogram is of continuously changing composition such that the eluting power of the solvent increases continuously. Since the rear of each chromatographic zone is in a stronger eluting medium than the front the result is a considerable sharpening of the zones. Moore & Stein (personal communication) have found that particularly good separation of amino-acid mixtures can be obtained on 4% cross-linked Dowex 50 resin columns with sodium citrate buffers and continuous increase in both pH and molarity.

For the fractionation of peptide mixtures, gradient elution is also valuable. We have obtained fairly good fractionation of a partial hydrolysate of casein, using 12% cross-linked Dowex 50 and gradient elution with ammonium formate buffers. Ammonium formate has the advantage over sodium citrate that it is volatile and can be removed from the effluent by vacuum sublimation (Hirs, Moore & Stein, 1952). Peptides were isolated from the effluent as DNP-peptides after further purification on silica columns (Askonas *et al.* 1954). More recently, greatly improved fractionation has been achieved on a new polystyrene resin (Zeo-Karb 225 (W.R. 1.1); 200–400 mesh) with a lower cross-linking (about 6%). Gradient elution was used with formate buffer over the range 0.2 M, pH 2.5 to 2.0 M, pH 5.5. Peptides from a partial acid hydrolysate of β-lactoglobulin were first filtered through charcoal to remove any material likely to be irreversibly adsorbed on the resin and then 300 mg. of material were fractionated on a column 150 cm. × 3 cm. Peptides were spread over about 500 fractions of effluent (60 ml. each) but each individual peptide was confined to about five fractions and some were concentrated in only two fractions. Peaks were symmetrical and showed little evidence of tailing.

4. Conclusion

The number and variety of peptides is so large that no one chromatographic method can be applicable to every problem of peptide fractionation. Nevertheless, from the above review a few generalizations do emerge. In the first place, relatively simple mixtures of large peptides are best fractionated either by counter-current methods or on ion-exchange resins. Secondly, a mixture of the complexity obtained by partial hydrolysis of protein can best be fractionated by successive application of two or more chromatographic methods, each depending on an entirely different set of physical properties. Thus, for example, fractionation by ion exchange can be followed by a partition method and this if necessary by an adsorption method. By suitable choice of chromatographic method even the most complex mixtures can be resolved. Up to the present, research on peptides has been limited by technical difficulties, but with these difficulties resolved immense fields are opened—the fields of protein structure, protein specificity and protein synthesis.

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SEPARATION OF CARBOHYDRATES AND PHOSPHORIC ESTERS ON PAPER CHROMATOGRAMS

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1 Carbohydrates
- *a* Mechanism of the paper chromatogram
- *b* Solvents
- *c* Movement of sugars on paper chromatograms
- *d* Spray reagents
- *e* Quantitative analysis

2 Phosphoric esters
- *a* Detection of phosphoric esters
- *b* Solvents
- *c* Filter-paper and its purification
- *d* Quantitative analysis

References

1. Carbohydrates

The separation of the simple sugars on the paper chromatogram was first described by Partridge (1948) and since then developments in the field have been unceasing. The method has been applied to the separation of the methylated sugars (Brown, Hirst, Hough, Jones & Wadman, 1948), the glycosides (Bate-Smith & Westall, 1950; Schindler & Reichstein, 1951) and of the oligosaccharides, both free (Dedonder, 1952; Edelman & Bacon, 1951) and as their *N*-benzylglycosylamine compounds (Bayly & Bourne, 1953). The present article describes the main features of the method, emphasis being placed on semi-empirical generalizations which summarize the results of the numerous published papers on the subject and allow a worker new to the field to select such solvents, sprays and quantitative methods as will yield a diagnostic result with the least number of experiments.

a. Mechanism of the Paper Chromatogram

The most important feature of chromatographic systems that comprise a hydrophilic solid and a solvent containing some proportion of water percolating through the solid is that the stationary phase must consist of the solid with a quantity of imbibed water. In the case of the paper chromatogram the solid phase will consist of cellulose with imbibed water. It seems quite clear that the water attached to the cellulose is different in many properties from liquid water and must exist with a structural organization based on the hydrogen bonding of the water molecules to the hydrophilic hydroxyl groups of the cellulose (Hanes & Isherwood, 1949). The water content of the water–cellulose complex in equilibrium with different flowing solvents may vary considerably but this will depend not upon whether particular solvents are miscible or immiscible with water, but upon their respective affinities for water. In the presence of mixed solvents such as aqueous methanol, ethanol, propanol, and acetone, cellulose is capable of absorbing water preferentially to give a water-enriched gel phase. It is probable that with the majority of solvents used for carbohydrates the cellulose is almost entirely covered with a layer of water molecules, and then the chromatographic system, with solutes such as sugars which have no special affinity for cellulose, approximates to that expected on the basis of a liquid–liquid chromatogram. However, it seems preferable to keep in mind that the stationary phase is basically a water–cellulose complex and that, in the case of certain solutes, the influence of the cellulose may be paramount. Recently Dalgliesh (1952) has shown that some aromatic amino acids—DL-kynurenine, 3-hydroxy-DL-kynurenine, 2:3- and 2:5-dihydroxyphenyl-DL-alanine—can be resolved on paper, using an optically inactive solvent. The solvent used (the organic phase of a mixture of *n*-butanol–acetic acid–water) is one that is popular with carbohydrate chemists. Somewhat similar observations have been made by Roberts & Wood (1953) on the catechins. The solvent in this case was water. It is clear that the resolution must be due to the intervention of the optically active cellulose in the chromatographic system. The problem is to describe the mechanism of the paper chromatogram in sufficiently general terms so that its future development as a device for separating solutes is not restricted by the ideas thus formulated. As a working hypothesis, Hanes & Isherwood (1949) envisaged that a solute molecule, by virtue of its hydrophilic character, is able to become incorporated competitively with water molecules (and solvent molecules) into the mobile but organized water–cellulose complex. This would involve the linking of the hydrophilic groups of the solute molecule to water molecules which are themselves already bound in the complex, and perhaps the occasional linking of a solvent molecule directly with a hydroxyl group of the cellulose. The separation of different solutes depends upon specific differences in their distribution between the water–cellulose complex on the one hand and the water-containing solvent on the other hand; and is a reflection of the ease with which the solute molecules enter into the mobile but organized water structure of each. It is clear that factors such as size and shape of the molecule, and the number, position, and character of the hydrophilic groups, will be the determinants of the specific distribution of solutes between the two phases in a given system.

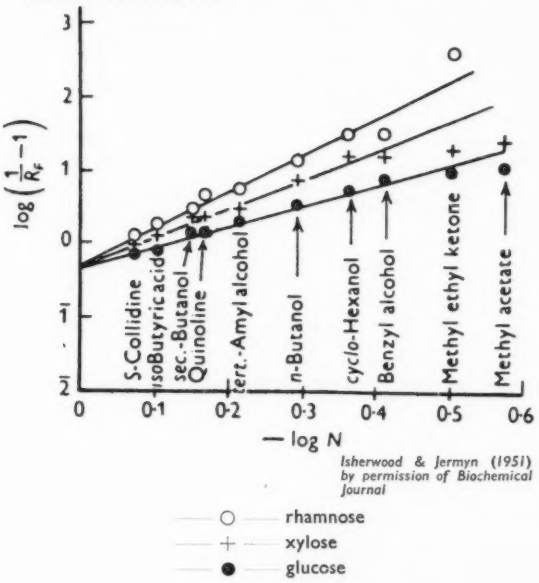
The sugars represent a special case because there is considerable evidence which suggests (Isherwood & Jermyn, 1951) that in solution they are heavily hydrated and that they behave to some extent like an association of water molecules. This would appear to be true of their movement on the paper chromatogram, for it has been shown that the distribution of any particular sugar between the two phases is related mainly to the affinity of the organic solvent for water molecules. This is true for the water-saturated solvents (except phenols) commonly in use. The relationship is shown in fig. 1, on which the graph of $\log\left(\frac{1}{R_F}-1\right)$ has been plotted against $-\log N$ (where N is the molar fraction of water in the solvent at 20° C.) for the three sugars, rhamnose, xylose and glucose. These sugars were selected because their R_F values covered the range of R_F values obtained for the simple sugars.

Each of the sugars gives a characteristic linear relationship which does not cross that for any of the other sugars except at the origin. This means that the order in which the sugars separate will be the same with all the solvents mentioned in fig. 1.

b. Solvents

For the general chromatography of the sugars a variety of solvents have been used by individual workers (cf. review by

FIG. 1. RELATIONSHIP BETWEEN R_F VALUE AND MOLAR FRACTION OF WATER (N) IN NON-AQUEOUS PHASE



Isherwood & Jermyn (1951) by permission of Biochemical Journal

Dedonder, 1952), though the actual order of separation is very much the same in all non-phenolic solvents. In fact, examination of an unknown sugar by the use of one of these solvents is sufficient to cover the whole group. Phenol, however, gives a different pattern of separation and can be used to provide an additional chromatographic criterion as to the identity of an unknown sugar. A further criterion can be obtained in the case of certain sugars by the addition of boric acid to the solvent. Boric acid readily complexes with *cis*-hydroxyl groups to give a hydrophilic compound and, when this is formed, the R_F value changes markedly. In practice it is found that only a few sugars are affected but, where the method does apply, it furnishes a valuable criterion as to the identity of the sugar (cf. identification of apiose, by Bell, Isherwood & Hardwick (1954), and of the 3-, 5-, and 3:5-methyl ethers of D-ribose by Barker & Smith, 1954).

Apart from changing the type of solvent, there is another method of discriminating between similar sugars. This relies on operating the chromatogram under conditions which effect the greatest possible separation of the solutes. Jermyn & Isherwood (1949) have shown that this occurs if solvents are used which give R_F values less than 0.2, the solvent being allowed to run off the end of the paper until the sugars have moved 10–20 cm. The main drawback to this method is that the time required for the spots to move apart on the paper can be considerable if the solvents used have a high viscosity, e.g. *cyclo*hexanol. It is essential, therefore, to use solvents that have a low viscosity in order that the solvent front may move as quickly as possible.

The information derived from a study of the chromatograms can be meaningless if an appreciable quantity of salts is present in the original sugar solution. To overcome this difficulty, which is very likely to be present with biological extracts, five methods have been tried.

i. The salts can be removed by the use of ion-exchange resins. It is preferable to use weak base resins for the removal of the anions, as the strongly basic resins such as Amberlite IRA-400 and Dowex 2 are known to cause profound decomposition of certain sugars (Roseman, Abeles & Dorfman, 1952; Hulme, 1953).

ii. Since most carbohydrates are neutral molecules, salts can be removed in many cases by electrodialysis. A convenient apparatus has been described by Consden, Gordon & Martin (1947).

iii. The salts can be separated from the sugars by extracting the concentrated sugar solution with pyridine; the sugars dissolve in the pyridine and the inorganic salts are precipitated (Malpress & Morrison, 1949). The danger in this method is that pyridine readily epimerizes reducing sugars.

iv. In the case of enzyme experiments it is an advantage to use buffers that can be easily removed (phosphate can be removed by a preliminary treatment with $ZnSO_4$ and $Ba(OH)_2$ (Somogyi, 1945)).

v. The difficulty can be largely overcome if solvents such as *n*-butanol, or phenol, saturated with water and oxalic acid, are used (Chen, Isherwood & Mapson, 1953). The swamping concentration of electrolyte minimizes the effect of the salts, and the solutions can be chromatographed directly. This is particularly useful when labile carbohydrates (e.g. L-ascorbic acid) are present, which might be destroyed by the preliminary salt-removing techniques.

c. Movement of Sugars on Paper Chromatograms

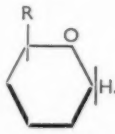
A comprehensive knowledge of the R_F values of the sugars in at least one solvent has a value beyond the mere figures. The fact that the sugars separate in the same order in all non-phenolic solvents means that if the R_F values are known for one solvent, then the same order of R_F values applies to all. In addition, since the sugars of one class have very similar structures and yet separate in the same order in all non-phenolic solvents, it seems probable that the R_F values are related to the disposition of the hydroxyl groups round the molecule. If some systematic relationship were discerned between the structure of the sugars and their R_F values, it might be possible to predict the structure of an unknown sugar merely from an observation of its R_F value. It was with this objective in mind that Isherwood & Jermyn (1951) made an accurate measurement of the R_F values of a large number of carbohydrates, using as solvent the water-poor phase of a two-phase mixture of ethyl acetate–pyridine–water (2:1:2 vol. respectively).

A brief survey of their figures (Table I) indicates that while the number of hydroxyl groups in a monosaccharide has some relation to the R_F value, other factors contribute largely to the observed results. Grouping the sugars on the basis of the number of hydroxyl groups alone means, in the case of the pentoses (0.23–0.37), hexoses (0.175–0.31) and heptoses (0.14–0.23), that the range of R_F values in each group considerably overlaps those in the other groups. The oligosaccharides, however, give a more definite relationship. The R_F values progressively decrease as the number of sugar residues in the molecule increases.

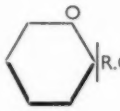
A more systematic study elucidated the fact that the R_F values were related to the disposition of the hydroxyl groups on the ring form of the sugars. In Table I, the sugars have been arranged in groups on the basis of the configuration of the atoms which compose their ring forms, and the R_F values

TABLE I. RELATIONSHIP BETWEEN THE CONFIGURATIONS AND THE R_F VALUES OF SUGARS, GLYCOSIDES AND γ-LACTONES IN ETHYL ACETATE–PYRIDINE–WATER AT 20° C.

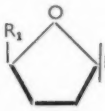
(The configuration of the sugars is described according to the Fischer convention, thus D-glucose $HOCH_2$—C(H)—C(H OH)—C(H)—C(H OH)—CHO is written + + − +. The configuration of the ring is shown in the bracketed column, e.g. + [+ − +]. The figures enclosed in square brackets are the R_F values in phenol.)

(i) Sugars with the general formula  where R = —CH_3, —CH_2OH, —COOH, —$CHOH.CH_2OH$

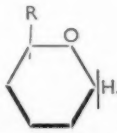
Configuration (ring)	Aldomethylose sugars R = —CH_3	m.p.(°C.)	R_F	Aldohexose sugars R = —CH_2OH	m.p.(°C.)	R_F	Aldohexuronic acids * R = —COOH	m.p.(°C.)	R_F	Aldoheptose sugars R = —$CHOH.CH_2OH$	m.p.(°C.)	R_F
+ + + +	Allomethylose	146	0.36 [0.68]	Allose	128	0.22	—	—	—	—	—	—
+ + + −	Altromethylose	—	0.45 [0.65]	Altrose	108	0.27	—	—	—	—	—	—
+ + − +	Glucomethylose	139–145	—	Glucose	146–150	0.195 [0.38]	Glucuronic	165	0.15 [0.18]	D-Gala-L-giucose	190–194	0.1
+ − + +	Gulomethylose	—	0.36	Gulose	—	0.23	—	—	—	D-Gluco-D-gulose	210	0.1
+ + − −	Mannomethylose	123	0.38 [0.62]	Mannose	132	0.24 [0.46]	Mannuronic	165	0.18 [0.225]	D-Gala-L-mannose	—	0.2
+ − + −	Idomethylose	—	0.51	Idose	—	0.31 [0.25]	—	—	—	—	—	—
+ − − +	Galactomethylose	145	0.265 [0.64]	Galactose	165	0.175 [0.44]	Galacturonic	160	0.13 [0.19]	D-Gulo-L-galactose	—	0.1
										D-Manno-D-galactose	134	0.1
+ − − −	Talomethylose	—	0.48	Talose	128	0.285	—	—	—	D-Gulo-L-talose	—	0.2

(ii) Sugars with the general formula  where R = —H, —CH_2OH

Configuration (ring)	Ketohexose sugars R = —CH_2OH	m.p.(°C.)	R_F	Aldopentose sugars R = —H	m.p.(°C.)	R_F
+ + +	Allulose	(Syrup)	0.30	Ribose	95	0.33 [0.60]
+ − −	Tagatose	124	0.27	Lyxose	106–117	0.30 [0.52]
+ − +	Sorbose	164	0.24 [0.42]	Xylose	145	0.28 [0.46]
+ + −	Fructose	102	0.24 [0.52]	Arabinose	159	0.23 [0.56]

(iii) Sugars with general formula  where R = —H, —CH_2OH and R_1 = —H, —CH_3

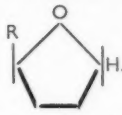
Configuration (ring)	Aldotetrose sugars R = R_1 = —H	R_F	Ketopentose sugars R = —CH_2OH, R_1 = H	R_F	Ketohexomethylose sugars R = —CH_2OH, R_1 = —CH_3	R_F
+ +	Erythrose	0.41	Araboketose	0.35	+ + + Allomethyloketose	0.44
					+ − − Galactomethyloketose	0.43
+ −	Threose	0.43	Xyloketose	0.37	+ + − Mannomethyloketose	0.43
					+ − + Idomethyloketose	0.47

(iv) Glycosides with general formula  where R = —H, —CH_2OH

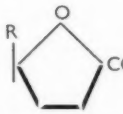
Configuration (ring)	Aldohexose glycosides R = —CH_2OH	R_F	Aldopentose glycosides R = —H	R_F
+ + + +	Allose	0.23	Ribose	0.40
+ + + −	Altrose	0.36	Arabinose	—
+ + − +	Glucose	0.29	Xylose	0.46
+ − + +	Gulose	—	—	—
+ + − −	Mannose	0.37	Lyxose	0.48
+ − + −	Idose	—	—	—
+ − − +	Galactose	0.34	—	—
+ − − −	Talose	0.29	—	—

* Indicates that these carbohydrate classes were run in ethyl acetate–acetic acid–water as solvent (Jermyn & Isherwood, 1949).

TABLE I (*continued*). RELATIONSHIP BETWEEN THE CONFIGURATIONS AND THE R_F VALUES OF SUGARS, GLYCOSIDES AND γ-LACTONES IN ETHYL ACETATE–PYRIDINE–WATER AT 20° C.

(v) Glycosides with general formula  where R = —CH_2OH, —$CHOH.CH_2OH$

Configuration (ring)	Aldohexose glycosides R = —$CHOH.CH_2OH$	R_F	Aldopentose glycosides R = —CH_2OH	R_F
+ + +	Allose	0.45	Ribose	0.54
+ + −	Altrose	0.48	Arabinose	0.55
+ − +	Glucose	0.43	Xylose	0.52
− + +	Gulose	—	—	—
+ − −	Mannose	0.48	Lyxose	0.55
− + −	Idose	—	—	—
− − +	Galactose	0.47	—	—
− − −	Talose	0.44	—	—

(vi) Lactones with general formula  where R = —CH_2OH, —$CHOH.CH_2OH$

Configuration (ring)	Aldohexonic γ-lactones * R = —$CHOH.CH_2OH$	m.p.(°C.)	R_F	Aldopentonic γ-lactones * R = —CH_2OH	m.p.(°C.)	R_F
+ + +	Allono	130	0.20 [0.48]	Ribono	78	0.27 [0.57]
+ + −	Altrono	—	0.21 [0.50]	Arabino	99	0.27 [0.565]
+ − +	Glucono	135	— [0.49]	Xylono	103	0.30 [0.58]
− + +	Gulono	185	0.15 [0.52]	—	—	—
+ − −	Mannono	152	0.18 [0.52]	Lyxono	110	0.22 [0.61]
− + −	Idono	—	0.20 [0.47]	—	—	—
− − +	Galactono	133	0.19 [0.47]	—	—	—
− − −	Talono	138	0.19 [0.45]	—	—	—

* Indicates that these carbohydrate classes were run in ethyl acetate–acetic acid–water as solvent (Jermyn & Isherwood, 1949).

are given alongside. The ring forms were assumed to be pyranose except where the chemical structure of the sugar prevented this being formed. Thus the pyranose forms of D-xylose, D-glucose, D-glucomethylose and L-gala-D-gluco-heptose all belong to the D-glucose type. These sugars differ only in the group attached to carbon atom 5 of the pyranose ring, the configuration of the atoms of the ring being the same in each case. Such a grouping of sugars has been termed homomorphous (Hann, Merrill & Hudson, 1935) and, as would be expected from the identity of the configuration of the ring, members of each homomorphous series often show marked chemical and physical similarities with each other. In such a series it is often possible to predict the properties of an unknown member from those of the basic type.

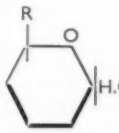
Comparison between the various groups of sugars in Table I shows that the R_F values of corresponding members of each group are markedly dependent on the configuration of the ring. In addition, the residue attached to either carbon atom 5 of the pyranose ring or 4 of the furanose ring exercises the same general influence on the R_F value independent of the configuration of the ring. A similar effect is observed with the residue attached to the reducing end of the ring. Comparison of the ketohexose and aldopentose sugars in Table I (ii) shows that replacement of —H on carbon atom 1 of any of the aldopentose sugars by —CH_2OH changes the R_F value by −10%. The sugars which can form only furanose rings have much higher R_F values than those which can form pyranose, and the differences between individual sugars are less. Reference to Table I (iv) and (v) shows that the range of R_F values for the pyranosides (0.23–0.37) is wider and the mean value (0.31) smaller than for the corresponding furanosides (0.43–0.48 and 0.46) respectively.

The fact that the sequence of R_F values for each class of sugars (aldohexose, aldomethylose, etc.) depends only on the configuration of the hydroxyls of the ring also appears to be true for the methyl glycosides, though the order of R_F values for each class of glycosides is not the same as for the parent sugars. D-Galactose, which has the lowest R_F value of the aldohexoses, gives a methyl pyranoside which has a R_F value well above the lowest of its class.

In Table I, the melting-points of a number of sugars have been included for comparison with the R_F values. In the case of the simple pentoses and hexoses and perhaps in the case of the γ-lactones, the R_F value is roughly inversely proportional to the melting-point. The link between the two properties probably lies in the affinity of the hydroxyl groups of the sugar for other hydroxyl groups; in the crystal the melting-point is a measure of the strength of the internal bonds between neighbouring sugar molecules which are largely held together by links between their hydroxyl groups, and in solution the R_F value is a measure of the affinity of the sugar for water molecules. The relationship will apply only if the association of a sugar with water molecules in solution is of the same nature and extent as the association of the hydroxyls of neighbouring sugar molecules in the solid.

TABLE I. RELATIONSHIP BETWEEN THE CONFIGURATIONS AND THE R_F VALUES OF SUGARS, GLYCOSIDES AND γ-LACTONES IN ETHYL ACETATE–PYRIDINE–WATER AT 20° C.

(The configuration of the sugars is described according to the Fischer convention, thus D-glucose $HOCH_2$—C(H)—C(H OH)—C(H)—C(H)—CHO is written + + − +. The configuration of the ring is shown in the bracketed column, e.g. + (+ − +). The figures enclosed in square brackets are the R_F values in phenol.)

(i) Sugars with the general formula  where R = —CH_3, —CH_2OH, —COOH, —$CHOH.CH_2OH$

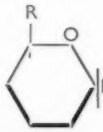
Configuration (ring)	Aldomethylose sugars R = —CH_3	m.p.(°C.)	R_F	Aldohexose sugars R = —CH_2OH	m.p.(°C.)	R_F	Aldohexuronic acids * R = —COOH	m.p.(°C.)	R_F	Aldoheptose sugars R = —$CHOH.CH_2OH$	m.p.(°C.)	R_F
+ + + +	Allomethylose	146	0.36 [0.68]	Allose	128	0.22	—	—	—	—	—	—
+ + + −	Altromethylose	—	0.45 [0.65]	Altrose	108	0.27	—	—	—	—	—	—
+ + − +	Glucomethylose	139–145	—	Glucose	146–150	0.195 [0.38]	Glucuronic	165	0.15 [0.18]	D-Gala-L-glucose	190–194	0.16
+ − + +	Gulomethylose	—	0.36	Gulose	—	0.23	—	—	—	D-Gluco-D-gulose	210	0.19
+ + − −	Mannomethylose	123	0.38 [0.62]	Mannose	132	0.24 [0.46]	Mannuronic	165	0.18 [0.225]	D-Gala-L-mannose	—	0.21
+ − + −	Idomethylose	—	0.51	Idose	—	0.31 [0.25]	—	—	—	—	—	—
+ − − +	Galactomethylose	145	0.265 [0.64]	Galactose	165	0.175 [0.44]	Galacturonic	160	0.13 [0.19]	D-Gulo-L-galactose	—	0.14
										D-Manno-D-galactose	134	0.15
+ − − −	Talomethylose	—	0.48	Talose	128	0.285	—	—	—	D-Gulo-L-talose	—	0.23

(ii) Sugars with the general formula where R = —H, —CH_2OH

Configuration (ring)	Ketohexose sugars R = —CH_2OH	m.p.(°C.)	R_F	Aldopentose sugars R = —H	m.p.(°C.)	R_F
+ + +	Allulose	(Syrup)	0.30	Ribose	95	0.33 [0.60]
+ − −	Tagatose	124	0.27	Lyxose	106–117	0.30 [0.52]
+ − +	Sorbose	164	0.24 [0.42]	Xylose	145	0.28 [0.46]
+ + −	Fructose	102	0.24 [0.52]	Arabinose	159	0.23 [0.56]

(iii) Sugars with general formula where R = —H, —CH_2OH and R_1 = —H, —CH_3

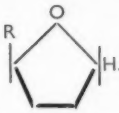
Configuration (ring)	Aldotetrose sugars R = R_1 = —H	R_F	Ketopentose sugars R = —CH_2OH, R_1 = H	R_F	Ketohexomethylose sugars R = —CH_2OH, R_1 = —CH_3	R_F
+ +	Erythrose	0.41	Araboketose	0.35	+ + + Allomethyloketose	0.44
					+ − − Galactomethyloketose	0.43
+ −	Threose	0.43	Xyloketose	0.37	+ + − Mannomethyloketose	0.43
					+ − + Idomethyloketose	0.47

(iv) Glycosides with general formula  where R = —H, —CH_2OH

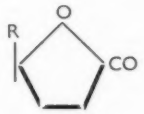
Configuration (ring)	Aldohexose glycosides R = —CH_2OH	R_F	Aldopentose glycosides R = —H	R_F
+ + + +	Allose	0.23	Ribose	0.40
+ + + −	Altrose	0.36	Arabinose	—
+ + − +	Glucose	0.29	Xylose	0.46
+ − + +	Gulose	—	—	—
+ + − −	Mannose	0.37	Lyxose	0.48
+ − + −	Idose	—	—	—
+ − − +	Galactose	0.34	—	—
+ − − −	Talose	0.29	—	—

* Indicates that these carbohydrate classes were run in ethyl acetate–acetic acid–water as solvent (Jermyn & Isherwood, 1949).

TABLE I (*continued*). RELATIONSHIP BETWEEN THE CONFIGURATIONS AND THE R_F VALUES OF SUGARS, GLYCOSIDES AND γ-LACTONES IN ETHYL ACETATE–PYRIDINE–WATER AT 20° C.

(v) Glycosides with general formula  where R = —CH_2OH, —$CHOH.CH_2OH$

Configuration (ring)	Aldohexose glycosides R = —$CHOH.CH_2OH$	R_F	Aldopentose glycosides R = —CH_2OH	R_F
+ + +	Allose	0.45	Ribose	0.54
+ + −	Altrose	0.48	Arabinose	0.55
+ − +	Glucose	0.43	Xylose	0.52
− + +	Gulose	—	—	—
+ − −	Mannose	0.48	Lyxose	0.55
− + −	Idose	—	—	—
− − +	Galactose	0.47	—	—
− − −	Talose	0.44	—	—

(vi) Lactones with general formula  where R = —CH_2OH, —$CHOH.CH_2OH$

Configuration (ring)	Aldohexonic γ-lactones * R = —$CHOH.CH_2OH$	m.p.(°C.)	R_F	Aldopentonic γ-lactones * R = —CH_2OH	m.p.(°C.)	R_F
+ + +	Allono	130	0.20 [0.48]	Ribono	78	0.27 [0.57]
+ + −	Altrono	—	0.21 [0.50]	Arabino	99	0.27 [0.565]
+ − +	Glucono	135	— [0.49]	Xylono	103	0.30 [0.58]
− + +	Gulono	185	0.15 [0.52]	—	—	—
+ − −	Mannono	152	0.18 [0.52]	Lyxono	110	0.22 [0.61]
− + −	Idono	—	0.20 [0.47]	—	—	—
− − +	Galactono	133	0.19 [0.47]	—	—	—
− − −	Talono	138	0.19 [0.45]	—	—	—

* Indicates that these carbohydrate classes were run in ethyl acetate–acetic acid–water as solvent (Jermyn & Isherwood, 1949).

are given alongside. The ring forms were assumed to be pyranose except where the chemical structure of the sugar prevented this being formed. Thus the pyranose forms of D-xylose, D-glucose, D-glucomethylose and L-gala-D-glucoheptose all belong to the D-glucose type. These sugars differ only in the group attached to carbon atom 5 of the pyranose ring, the configuration of the atoms of the ring being the same in each case. Such a grouping of sugars has been termed homomorphous (Hann, Merrill & Hudson, 1935) and, as would be expected from the identity of the configuration of the ring, members of each homomorphous series often show marked chemical and physical similarities with each other. In such a series it is often possible to predict the properties of an unknown member from those of the basic type.

Comparison between the various groups of sugars in Table I shows that the R_F values of corresponding members of each group are markedly dependent on the configuration of the ring. In addition, the residue attached to either carbon atom 5 of the pyranose ring or 4 of the furanose ring exercises the same general influence on the R_F value independent of the configuration of the ring. A similar effect is observed with the residue attached to the reducing end of the ring. Comparison of the ketohexose and aldopentose sugars in Table I (ii) shows that replacement of —H on carbon atom 1 of any of the aldopentose sugars by —CH_2OH changes the R_F value by —10%. The sugars which can form only furanose rings have much higher R_F values than those which can form pyranose, and the differences between individual sugars are less. Reference to Table I (iv) and (v) shows that the range of R_F values for the pyranosides (0.23–0.37) is wider and the mean value (0.31) smaller than for the corresponding furanosides (0.43–0.48 and 0.46) respectively.

The fact that the sequence of R_F values for each class of sugars (aldohexose, aldomethylose, etc.) depends only on the configuration of the hydroxyls of the ring also appears to be true for the methyl glycosides, though the order of R_F values for each class of glycosides is not the same as for the parent sugars. D-Galactose, which has the lowest R_F value of the aldohexoses, gives a methyl pyranoside which has a R_F value well above the lowest of its class.

In Table I, the melting-points of a number of sugars have been included for comparison with the R_F values. In the case of the simple pentoses and hexoses and perhaps in the case of the γ-lactones, the R_F value is roughly inversely proportional to the melting-point. The link between the two properties probably lies in the affinity of the hydroxyl groups of the sugar for other hydroxyl groups; in the crystal the melting-point is a measure of the strength of the internal bonds between neighbouring sugar molecules which are largely held together by links between their hydroxyl groups, and in solution the R_F value is a measure of the affinity of the sugar for water molecules. The relationship will apply only if the association of a sugar with water molecules in solution is of the same nature and extent as the association of the hydroxyls of neighbouring sugar molecules in the solid.

TABLE II. THE PROPERTIES OF VARIOUS SPRAY REAGENTS

(+ positive reaction ; — no reaction. Blank space signifies that reaction has not been described. The colours produced with different classes of sugars are indicated. Arabic numerals in brackets denote references.)

Spray reagent	Reaction given by various classes of sugar															Comments	Reference
	Aldohexose	Aldopentose	Ketohexose	Ketopentose	Uronic acid	Methyl pentose	Methylated aldohexose	Methylated ketohexose	Methylated aldopentose	Methylated uronic acid	Amino sugar	Sugar alcohols	Deoxy sugars	Lactones, esters, amides	Glycosides		
Class I																	
0.1 M-$AgNo_3$ in 5 M-NH_4OH. Heated at 100° C. for 5–30 min. Addition of 2 N-NaOH enables reagent to attack sugar alcohols, etc. Brown-black stains	+	+	+	+	+	+					+	±		±	±	This reagent is unspecific but is valuable because almost every compound on chromatogram either produces a stain or inhibits background colour. Sensitive to 0.001 M-glucose. An elegant modification of this reagent has been described (2) for reducing sugars	1
Alkaline solution of triphenyltetrazolium chloride reduced to give brilliant red triphenylformazan	+	+	+	+	+	+					+	—		—	—	In the author's hands this reagent was not sensitive to concentrations below 0.005 M-glucose	3
3:4-Dinitrobenzoic acid in ethanolic Na_2CO_3 reduced to 2-nitro-4-carboxylic phenylhydroxamine. Na salt, blue	+	+	+	+	+	+					+	—	+	—	—	Ketoses react more quickly than aldoses. Claimed to be as sensitive as the Ag reagent	4
3:5-Dinitrosalicylic acid in N-NAOH. Brown on yellow background	+	+	+	+	+	+					+	—	+	—	—	Sensitivity less than 0.01 M-glucose	5
$KMNO_4$ (1%) in 2% aqueous Na_2CO_3. No heating. Brown on purple background	+	+	+	+	+	+					+	±	+	±	±	Sensitivity less than 0.01 M-glucose. Unspecific and not permanent	6
Class II																	
Aniline phthalate (2%) in moist butanol (similarly its trichloroacetate or phosphate).	Brown	Red	—	—	Brown	Brown	Brown	Brown	Red	Red	—	—	—	—	—	Not quite as sensitive as the Ag reagent. Similar reagents based on diphenylamine, dimethylamine, α-naphthylamine and benzidine (9) have been described. React with aldoses	7
p-Anisidine hydrochloride (3%) in moist butanol	Greenish brown		Yellow		Red	Green	Brown		Red				Light brown				8
Naphthoresorcinol (0.1%) in 0.4 N-HCl in 80% ethanol		Bluish violet	Red		Blue	Bluish violet					—		—			Similar reagents based on orcinol and resorcinol (8) and anthraquinone. Sensitivity less than the Ag reagent. Reacts particularly with ketohexoses	10
Urea (5%) in 0.4 N-HCl in 80% ethanol	—	—	Intense brownish black	—	—			Brownish black								Reacts particularly with ketohexoses	11

References: 1. Partridge (1946); 2. Trevelyan, Proctor & Harrison (1950); 3. Wallenfels (1950); 4. Weygand & Hofmann (1950); 5. Jeanes, Wise & Dimler (1951); 6. Pacsu, Mora & Kent (1949); 7. Partridge (1948); 8. Hough, Jones & Wadman (1950); 9. Horrocks (1949); 10. Forsyth (1948); 11. Dedonder (1950)

In Table I, the R_F values of certain of the sugars in phenol are also recorded. No systematic classification of the R_F values of the substituted sugars analogous to those shown in Table I has yet been attempted (cf. Hirst & Jones, 1949a).

d. Spray Reagents

A large number of such reagents have been described though it is probable that only about six are in common use. In the assessment of the value of the various reagents two factors have to be considered: whether the reagent will give a distinct colour difference between compounds of two different classes, and the concentration of sugar on the chromatogram which can just be detected. In the present study the spray reagents mentioned in Table II have been tested by the author against various concentrations of sugars (0.01 M- and 0.001 M-glucose, fructose, rhamnose, arabinose, glucosamine, galacturonic acid, and 2-deoxyglucose) which were applied to the starting line of a paper chromatogram (Whatman No. 1 washed), and the paper run with the ethyl acetate–acetic acid–water solvent (Jermyn & Isherwood, 1949) for 17 hours. The comments in Table II give an approximate idea of the practical sensitivity of the reagents. It is easy to obtain an optimistic idea of the sensitivity if the testing is done against the same sugar solutions merely spotted on paper.

The reagents which are useful for a wide variety of sugars can be divided into two main classes.

i. The sugar reduces the reagent to give a coloured derivative.

ii. The sugar is heated with an acid so that furfuraldehyde, methylfurfuraldehyde, or ω-hydroxymethylfurfuraldehyde is produced; this, in the presence of an aromatic amine, or phenol, gives a characteristically coloured compound. Since both the rate at which the furfuraldehyde derivative is formed and the type are closely connected with the chemical nature of the sugar, it follows that different classes of sugars, and often different sugars of the same class, give different colour reactions.

A representative list of these reagents is given in Table II.

In addition to the reagents described above, there are a few that are specific to certain groupings in the molecule and are not applicable to a large number of carbohydrates. These are described separately.

The *enediol* compounds like ascorbic acid are revealed by spraying the paper with 2:6-dichlorophenolindophenol. On an acid paper they show as white spots on a pink background.

In order to detect esters, lactones, amides and methylamides of aldonic and uronic acids, the paper is sprayed with an alkaline ethanolic solution of hydroxylamine, and then, after a short interval, with an aqueous solution of $FeCl_3$ in dilute HCl. The Fe salts of the hydroxamic acids are blue-mauve in colour. The reagent is not very sensitive and concentrations below 0.01 M cannot be detected with certainty.

The amino sugars can be detected by spraying with ninhydrin, but a more specific reagent is that described by Morgan and Elson and used by Aminoff, Morgan & Watkins (1950) for the identification of sugars produced by hydrolysis of blood group A substance.

To detect the non-reducing carbohydrates (Buchanan, Dekker & Long, 1950), the paper is first sprayed with periodate and then, after a short interval, is treated with SO_2 gas until all the iodine set free is reduced, and finally sprayed with Schiff's reagent. Coloured spots reveal the presence of the carbohydrate. The periodate oxidizes the carbohydrate, giving aldehyde groups which react with Schiff's reagent. Carbohydrates containing the 1:2:3-triol system may be distinguished from the 1:2-diols because they also give formic acid. This can be revealed by spraying the paper with iodide after the excess periodate has been destroyed with ethylene glycol. A brown iodine stain is positive. Terminal deoxy sugars represent a special case because acetaldehyde is produced and this can be detected by reaction with sodium nitroprusside and a secondary amine. Other deoxy sugars may be detected by spraying with an acidic solution of *p*-nitroaniline. A deep yellow colour is produced.

An alternative method for detecting non-reducing carbohydrates is to spray the paper with a benzene solution of lead tetraacetate. An oxidation similar to that with periodate occurs. The carbohydrate is revealed as a white spot on a brown background.

Practical experience with the reagents described in Table II and with the more specific ones mentioned in the text indicates that it is preferable to run sugars of known constitution alongside the "unknowns" on the paper chromatogram because the colours obtained with the various spray reagents vary with the paper, solvent and traces of impurity present.

e. Quantitative Analysis

It was a logical sequence to the successful use of paper chromatography for the qualitative separation of sugars that attempts should be made to apply the method for quantitative analysis. At the start the ordinary methods for the determination of reducing sugars were applied and the amount of sugar required was often considerable (1,000 μg.) as compared with the amounts used in the qualitative separations (10–100 μg.). More recently methods have been developed which will analyse quantities of the order of 10–100 μg. with an accuracy better than ± 5%. The actual method chosen will depend upon the nature of the sugar—whether aldose, ketose or non-reducing—and upon the effectiveness of the separation from neighbouring sugars on the chromatogram. Direct photometric methods are applicable where the sugars are spaced out widely on the paper chromatogram. Some of the methods are specific and in suitable cases this allows a sugar to be estimated even when not completely separated from a sugar of a different type.

In all quantitative methods based on a preliminary separation on the paper chromatogram, it is helpful if the paper itself has been purified by washing so that no carbohydrate material is present which can be extracted along with the sugar, or which can react with the spray reagent if the sugar is to be estimated by direct photometry. (The washing of filter-paper is referred to later under phosphoric esters.) Such washed paper increases the sensitivity of many of the spray reagents because there is little or no background colour. This is particularly noticeable in the case of the ammoniacal silver nitrate reagent.

The solvent used should be free from any impurities likely to react in the quantitative method. If the traces of solvent that are normally adsorbed on the paper even after prolonged drying are also deleterious, it is an advantage to "steam" the paper. The presence of moisture displaces the adsorbed solvent (e.g., phenol or acetic acid) very rapidly.

With all the quantitative methods listed below, it is essential for work of the highest accuracy to calibrate the method by running a small series of known determinations with the unknowns.

A selection of the more interesting methods is given in Table III.

TABLE III. METHODS FOR THE QUANTITATIVE ANALYSIS OF SUGARS SEPARATED ON PAPER CHROMATOGRAMS
(Arabic numerals in brackets denote references.)

Methods used for locating sugar	Methods used for analysis of sugar	Recommended range (μg.)	Accuracy claimed (%)	Comments	Reference
Paper sprayed with alkaline triphenyltetrazolium chloride solution	Red formazan compound eluted from paper with pyridine containing 10% HCl, and estimated colorimetrically	5–100	—	Appears to be suitable for all reducing sugars, though it is less sensitive for aldoses than for fructose	1
Paper sprayed with aniline phthalate and heated at 105° C.	Paper photographed. Density of photographic image measured with a photometer. Suitable for aldoses	5–150	5	Inhomogeneity of paper makes it difficult to secure results comparable in precision to those obtained by analysis of eluates.	2
$AgNO_3$ added to flowing solvent, paper exposed to NH_3 and heated	Direct measurement of spot density using a densitometer. Suitable for all reducing sugars	5–30	5	Techniques are useful for rapid approximate analysis	3
Simultaneous * chromatography of a spot of same sugar mixture on chromatogram, sprayed and then compared	Sugars in appropriate piece of paper eluted with water. Estimated by oxidation with alkaline hypoiodite. (For aldoses only)	above 500	8	Applicable to methylated aldoses as well.	4
		40	5	Incomplete oxidation of a number of sugars: mannose, rhamnose, lyxose and talose. Ketoses do react slightly with reagent	5
Simultaneous * chromatography of a spot of same sugar mixture on chromatogram, sprayed and then compared	Oxidation of sugar in eluate with periodate at 100° C. Excess periodate destroyed with glycol. Formic acid present titrated with alkali	about 200	5	Applicable to reducing sugars, and to disaccharides and higher saccharides. Also glycosides	6
Simultaneous * chromatography of a spot of same sugar mixture on chromatogram, sprayed and then compared	Reaction of aldose sugar with aniline trichloroacetate in ethanol at 100° C. Estimated colorimetrically	20–300	5	This method has been inserted because it appears to the author to be very promising and, though it has been applied only to column effluents, should be applicable to paper chromatogram eluates (see 8)	7
Simultaneous * chromatography of a spot of same sugar mixture on chromatogram, sprayed and then compared	Alkaline copper reagent in combination with arsenomolybdate (colorimetric measurement of molybdenum blue). Reaction carried out under nitrogen. (For reducing sugars)	10–350	1 (at 100 μg.)	Earlier methods based on the same reagents (see 9, 16) were unreliable for small amounts of sugar, because of unpredictable variations in the blank value. Exclusion of oxygen improves the accuracy of the method considerably	9
			3 (at 10 μg.) (not on eluates from paper chromatograms)		10
Simultaneous * chromatography of a spot of same sugar mixture on chromatogram, sprayed and then compared	Eluate treated with glycerol, HCl, $CuSO_4$ and resorcinol. Estimated colorimetrically	10–200	5	Ketohexoses give 100–200 times the colour of aldoses	11
Simultaneous * chromatography of a spot of same sugar mixture on chromatogram, sprayed and then compared	Eluate treated with anthrone and concentrated H_2SO_4. Estimated colorimetrically	8–200	2 (presumably at 200 μg.)	A similar reagent based on phenol (12) is claimed not to be affected by traces of the usual solvents used in chromatography. All carbohydrates give a colour. The H_2SO_4 used must be colourless. A similar method (15) has been described using orcinol	13

References: 1. Wallenfels (1950); 2. Gustofsson, Sundman & Lindh (1951); 3. McFarren, Brand & Rutkowsky (1951); 4. Hirst, Hough & Jones (1949); 5. Hawthorne (1947); 6. Hirst & Jones (1949b); 7. Gardell (1951); 8. Blass, Macheboeuf & Nunez (1950); 9. Duff & Eastwood (1950); 10. Wager (1954); 11. Bell (1954); 12. Dubois, Gilles, Hamilton, Rebers & Smith (1951); 13. Morris (1948); 14. Boissonnas (1950); 15. Alm (1952); 16. Flood, Hirst & Jones (1947)

*** In certain cases the position of the sugar spot can be located by the technique of Boissonnas (see 14), in which the paper is spotted at regular intervals with aniline phthalate. Only a minute proportion of the sugar is affected.**

Certain methods have not been included in Table III because they relate only to compounds that have a characteristic chemical grouping in the molecule, such as ascorbic acid which can be estimated by the specific titration with 2:6-dichlorophenolindophenol (Chen *et al.* 1953).

2. Phosphoric Esters

In the past, analysis of these compounds in biological extracts has mainly depended either upon the fractionation of salts of the esters, or upon the selective hydrolysis of some of them under standard conditions. Both procedures have been supplemented when possible by methods based on more specific reactions given by the non-phosphoric moiety of particular esters. Except when applied to certain relatively simple mixtures, these methods have frequently failed to yield diagnostic results. This is hardly surprising when one considers that many of the esters have very similar structures and that a number of such esters may be found, either in a biological extract, or even in an isolated enzyme experiment.

The phosphoric esters are very different in some respects from any of the other groups of organic compounds which have been separated on paper chromatograms. They are strong acids (dissociation constant for first H^+, p*K ca* 1.0) and markedly hydrophilic. They are readily adsorbed by traces of heavy metals in the paper and in addition some of them are easily decomposed by exposure to strong alkali or acid. The nature of the organic moiety also varies very widely. These properties mean that the ordinary solvents commonly used for the chromatography of carbohydrates and amino acids are likely to be inadequate. New solvents and innovations in technique were, in fact, found to be essential.

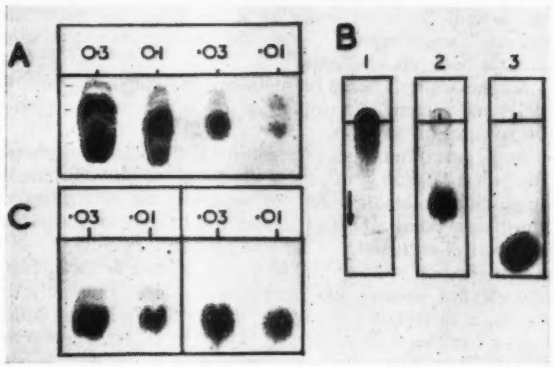
a. Detection of Phosphoric Esters

The method described by Hanes & Isherwood (1949) consists in spraying the chromatogram with an acid molybdate (1% in 3% perchloric acid) solution, followed by heating under conditions (10 min. at 80° C.) causing hydrolysis of the esters without undue decomposition of the paper itself. The free orthophosphoric acid produced from the esters forms a phosphomolybdate complex with the molybdate present, and this is reduced to an intensely blue compound by treating the paper with H_2S gas, the background becoming faint buff in colour due to the conversion of molybdate to molybdenum trisulphide. The treatment hydrolyses even such resistant esters as phosphoglyceric acid and glucose 6-phosphate, to render them detectable in minute amounts (e.g., 3 μl. of 0.001 M solution spread over 1 cm.² of paper).

Later workers have introduced a number of minor modifications. The chromatograms can be dipped into the reagent instead of sprayed, if part of the water (up to 85%) present in the original formula is replaced by acetone (Burrows, Grylls & Harrison, 1952). The phosphoric esters can be conveniently hydrolysed (even the resistant ones) by exposing the treated paper to ultra-violet light (Bandurski & Axelrod, 1951), and the final development of the molybdenum blue colour effected by spraying with a reducing agent such as ascorbic acid (Leuthardt & Testa, 1951) or an aminophenol in sodium bisulphite (Dedonder, 1952).

The phosphoric esters can also be hydrolysed enzymically with an alkaline phosphomonoesterase (Fletcher & Malpress, 1953).

FIG. 2. DEFECTS IN CHROMATOGRAMS OF PHOSPHORIC ESTERS



Hanes & Isherwood (1949) by permission of Nature

A: multiple-banded spots using the water-poor phase from butanol–water–acetic acid (40:50:10 vol.) as solvent
Spots: glucose 1-phosphate 0.3, 0.1, 0.03, 0.01 M as marked on the figure

B: fixation and trailing using propanol–water–ammonia (60:10:30 vol.) as solvent
Spots: 0.02 M-H_3PO_4
1: Whatman No. 1 paper as supplied
2: Whatman No. 54 paper (acid washed)
3: Whatman No. 1 paper washed in 0.1 N-HCl and water

C: "lagging shadows" using the water-poor phase from *tert.*-amyl alcohol–water–*p*-toluene–sulphonic acid (60:30 vol. + 2% by wt. of acid) as solvent
Spots: glucose 1-phosphate 0.03 and 0.01 M as marked on the figure
Left: Whatman No. 1 paper as supplied
Right: Whatman No. 1 paper washed in 1% aqueous ethanolic 8-hydroxyquinoline solution

Particular groups of phosphoric esters may be detected by spray reagents which are specific for the non-phosphoric moiety. Walker & Warren (1951) have developed a reagent for fructose esters based on naphthoresorcinol. Nucleotides can be detected by ultra-violet fluorescence.

b. Solvents

Various solvent systems were described by Hanes & Isherwood (1949), of which the following are most useful:

i. *Acid, water-immiscible solvents. tert.*-Amyl alcohol–water–90% formic acid (3:3:1 vol.).
ii. *Acid, water-miscible solvents. tert.*-Butanol–water–picric acid (8:2 vol. plus 4 g.); *iso*propyl ether–90% formic acid (3:2 vol.).
iii. *Basic, water-immiscible solvents.* Ethyl acetate–pyridine–water (20:9:20 vol.).
iv. *Basic, water-miscible solvents. n*-Propanol–concentrated ammonia–water (6:3:1 vol. respectively).

For separations on the acid side, rather strongly acid conditions were necessary in order to obtain movement of the esters as compact round spots. Under less acid conditions, for example with *n*-butanol–acetic acid–water mixtures, there was a tendency to form a multiple-banded spot of the type shown in fig. 2A. Tertiary alcohols were used to avoid any

esterification under the strongly acid conditions. Primary alcohols esterified under the same conditions so rapidly that the solvent deposited water as it ran down the paper and caused "waterlogging". On the alkaline side the most suitable base was ammonia.

Similar solvents based on methanol (Bandurski & Axelrod, 1951) and acetone (Burrows *et al.* 1952) have been suggested for solvents (ii) and (iv).

In the case of mixtures of adenosine and inosine phosphates, Deutsch & Nilsson (1953) use a solvent based on *iso*propanol–ammonium sulphate–water to separate the two series of phosphoric esters and then solvent (iv) to separate individual members of each series.

The addition of boric acid to neutral (Cohen & Scott, 1950) and alkaline solvents has been found to alter markedly the R_F values of certain sugar esters, those which presumably can form a complex with boric acid. Dr F. E. G. Harrap and the author have shown that the addition of boric acid to solvent (iv) enables glucose 1-phosphate to be readily separated from glucose 6-phosphate, whereas in the absence of boric acid they do not separate.

c. Filter-Paper and its Purification

Two serious defects in the chromatograms were traced to interference by inorganic impurities present in the filter-paper. Organic impurities present did not obviously interfere with the qualitative separation but would undoubtedly contaminate any phosphoric ester eluted from the paper.

The first defect, which was prominent in alkaline solvents (fig. 2B), was due to the presence of Ca or Mg in the paper. The main characteristic is that the movement of the spots is retarded. Washing the paper with dilute acids or with such complexing reagents as ethylenediaminetetraacetic acid removed this defect. The complexing reagent could also be added to the solvent.

The second defect appeared in acid solvents (fig. 2C). It takes the form of a "shadow" extending behind the moving spot and is probably due to more than one heavy metal. Washing the papers with 8-hydroxyquinoline eliminated this defect in most cases and in the others the addition of a little H_2S gas to the chromatogram jar was successful.

The most effective procedure for preparing purified filter-paper is to wash it chromatographically with dilute acid, water, and then with strong aqueous ammonia (Isherwood & Hanes, 1953). This treatment removes practically all the organic and inorganic impurities, though it may still be necessary to treat the paper with a reagent such as 8-hydroxyquinoline to inhibit the action of certain heavy metals.

d. Quantitative Analysis

An area of the filter-paper containing the spots is cut out, wet-ashed, and the phosphorus measured by the method of Berenblum & Chain (1938). For reliable results, the solvent must be free from aldehydes (Eggleston & Hems, 1952) and the phosphorus blank values for the paper must be low.

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PORPHYRINS

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1 Column chromatography
2 Paper chromatography
References

The use of chromatography for the separation of pyrrole pigments may be said to have begun with the technique itself, when in 1906 Tswett achieved the first separations of the chlorophylls on columns of $CaCO_3$. In many respects the separation of the porphyrins on columns is quite as simple as that of the chlorophylls, and it is an interesting reflection of the eccentricities of technical development that it was not until the 1930's that the method began to be applied effectively to porphyrins. As always, the development of the technique has revealed unsuspected complications, which in turn impose greater demands upon the methods. It is proposed to review briefly the methods now available, to point out some complications they are revealing, and to show how attempts are being made to resolve the difficulties.

The porphyrins with which this discussion will be chiefly concerned are the four isomeric uroporphyrins, each with eight carboxylic acid side-chains, the four isomeric coproporphyrins, each with four carboxylic side-chains, and protoporphyrin, which has two such side-chains. The structures of these, and the relationships between the isomers, are indicated in fig. 1. For further chemical details the reader must be directed to standard works such as that of Fischer & Orth (1937) or of Lemberg & Legge (1949). Of the nine compounds mentioned, isomers I and III of coproporphyrin and uroporphyrin, and isomer IX of protoporphyrin, are recognized as the most important porphyrins of natural occurrence.

1. Column Chromatography

Talc columns were used for purification of porphyrin esters by Fischer & Hofmann (1937), and for free porphyrins by Comfort (1949), who used aqueous HCl for the elution.

Columns of Al_2O_3 were used with some success for the separation of porphyrin esters by Waldenström (1935), Prunty (1946), Lederer & Tixier (1947) and McSwiney, Nicholas & Prunty (1950), while $CaCO_3$ columns were used by Grinstein, Schwartz & Watson (1945), Watson, Schwartz & Hawkinson (1945) and Gray & Holt (1948).

The technique was studied systematically by Nicholas (1951). The adsorbents Al_2O_3, MgO, $MgCO_3$ and $CaCO_3$ were each prepared in several grades of adsorptive power, classified by the method of Brockmann & Schodder (1941; cf. Williams, 1946); and the separation, upon such columns, of the methyl esters of uroporphyrin, coproporphyrin and protoporphyrin, and some other dicarboxylic porphyrins, was achieved with a variety of organic solvent mixtures. This study not only supplied very efficient methods for the separations, but also emphasized the fact that reproducible results can be obtained easily, provided that due attention is paid to the standardization of the adsorptive agents and to the purity of the solvents. On all columns, porphyrin esters separate in the order of the number of carboxylic side-chains they possess. Thus, on Al_2O_3 grade IV, the ease of elution decreases from protoporphyrin to coproporphyrin to uroporphyrin. On MgO grade III, on the other hand, the order of fastness of adsorption is reversed, ease of elution increasing from protoporphyrin to coproporphyrin to uroporphyrin. Preparations of porphyrin esters are sometimes contaminated with small amounts of the free (non-esterified) porphyrins, and such material remains, as an immobile band, at the top of all these columns. Small amounts of certain coloured degradation products of porphyrins are also very strongly adsorbed, and can thus be removed. The spectroscopic properties of these materials often suggest that they arise through oxidation of porphyrins.

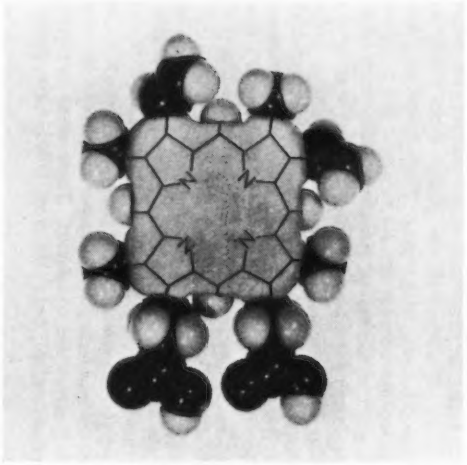
There has been little clear evidence of separation of porphyrin isomers by these columns, but Rimington's observation in 1936, that uroporphyrin isomers separate on Al_2O_3 under certain conditions, provides an important clue. Sümegi (1951) has claimed the separation of non-esterified coproporphyrins I and III on an Al_2O_3 column, developed with ether–acetic acid mixtures.

Most of the above are clearly adsorption chromatograms. Columns using partition chromatography have also been used. Lucas & Orten (1951) have separated porphyrin esters on columns of hydrated silica gel, developing with chloroform–ligroin mixtures. Dr J. J. Scott (personal communication) has used Celite columns in an interesting way. A well-known method for the purification of porphyrins depends upon their partition between ether and aqueous HCl (cf. Willstätter & Mieg, 1906). Scott has adapted this partition method to the chromatography of free porphyrins on Celite columns, using aqueous HCl as the fixed, and ether as the mobile phase. The sensitivity of these columns appears to be at least equal to the great sensitivity found by Granick & Bogorad (1953), who took advantage of the same partitioning behaviour to separate porphyrins by counter-current distribution.

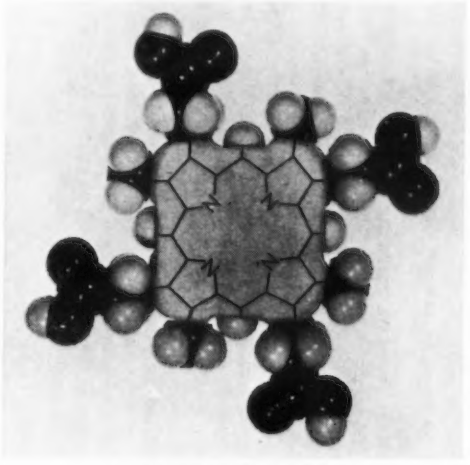
2. Paper Chromatography

Concurrently with their systematic studies of column methods, Nicholas & Rimington (1949, 1951) developed a descending paper chromatographic method for free porphyrins, using as solvent system the phases from mixtures of 2:4- and 2:5-lutidine with water. They found an inverse linear relationship between the R_F values of the porphyrins and the number of carboxylic side-chains. This method provides a penetrating qualitative tool which has been used extensively in conjunction with the more preparative column chromatography described in section 1. A few representative examples of the applications of such analyses to pathological material are to be found in the studies of Nicholas & Rimington (1951), McSwiney *et al.* (1950), Rimington & Miles (1951), Canivet, Fallot & Poidatz (1952), Canivet & Rimington (1953), and Macgregor, Nicholas & Rimington (1952). Details of most of the ancillary manipulations necessary may also be found in these papers. Further, many examples are to be found there of porphyrins which, on the paper chromatograms, have R_F values corresponding to unknown porphyrins possessing 3, 5, 6 or 7 carboxylic side-chains. These occur in relatively very small quantities; Grinstein *et al.* (1945) have, however,

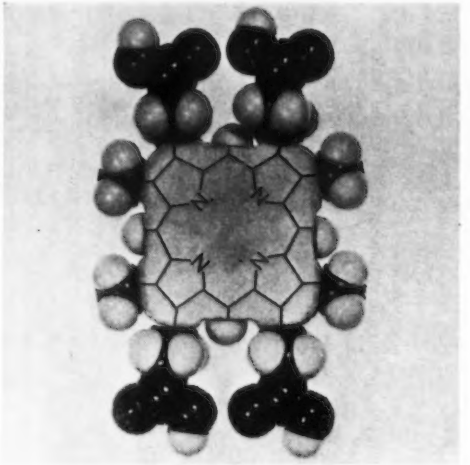
FIG. 1. SCALE MODELS OF SOME PORPHYRIN MOLECULES



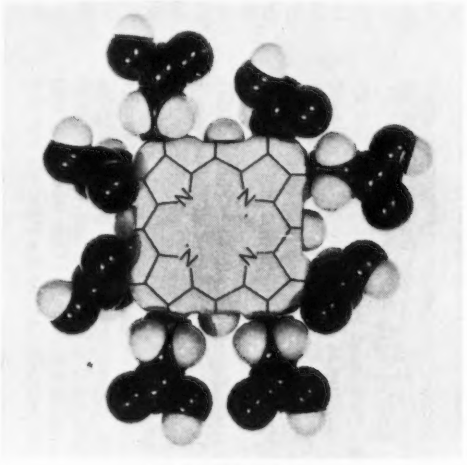
PROTOPORPHYRIN IX



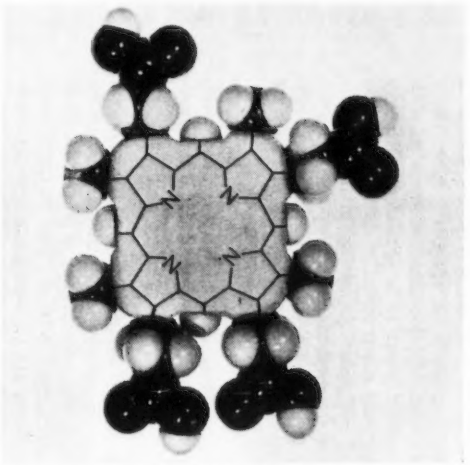
COPROPORPHYRIN I



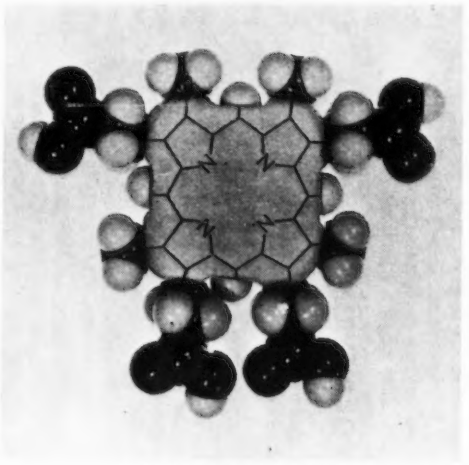
COPROPORPHYRIN II



UROPORPHYRIN III



COPROPORPHYRIN III



COPROPORPHYRIN IV

The dimensions of the planar porphin nucleus are based upon those of the metal-free phthalocyanine molecule (Robertson, 1936), the scale chosen being identical with that of the atomic models (Catalin Ltd., London) used for the side-chains. In the Catalin models, the cut faces provide for correct inter-atomic angles and distances, and the spherical dimensions show the van der Waals radii ; it is the latter which give the side-chains their bulky appearance. These models should represent fairly accurately the true stereochemistry of the porphyrins, the most important inaccuracy probably being in the bond angles at the methene bridge carbons ; these would be expected to be more obtuse than the C=N–C angles of phthalocyanine which are shown. Greater precision will not be possible until the full *x*-ray crystallographic analysis of a porphyrin has been achieved.

The side-chains in the coproporphyrins are methyl and propionic acid groups ; in the uroporphyrins all the methyl groups are replaced by acetic acid groups, and in protoporphyrins two of the propionic acid residues of coproporphyrin are replaced by vinyl groups.

been able to isolate a porphyrin, the elementary analysis of which indicated a heptacarboxylic porphyrin. These porphyrins often move, on the column chromatograms used for their isolation, in an intermediate way consistent with their behaviour on the paper chromatograms.

More recently, Eriksen (1953) has introduced a method based upon that of Nicholas & Rimington (1949, 1951), but using the ascending technique and a mixture of 2:6-lutidine and water. This method is very convenient; 2:6-lutidine gives a single-phase system with water, and the method is not so sensitive to small temperature changes as the earlier one. The relationship between R_F and number of carboxylic side-chains was similar to that found by Nicholas and Rimington.

Miss A. Benson has recently found, in the writer's laboratory, that, with a slight modification, Eriksen's method is capable of separating certain isomers. The four isomers of coproporphyrin were tested: isomers II and IV from the collection of the late Hans Fischer and courteously supplied to us by Professor S. Goldschmidt, isomer I prepared from calf meconium and isomer III from culture filtrates of *Corynebacterium diphtheriae*. Isomers I and II moved respectively slower and faster than isomers III and IV, the two latter having identical R_F values. It was then found that the method of Nicholas and Rimington also is capable of separating the coproporphyrin isomers. This finding is a very important one, since it provides the first lead we have had to conditions which may, with further modification, allow the separation of all four isomers. It is important in another sense, however; it must render more difficult the interpretation of certain spots which sometimes appear on these chromatograms. Coproporphyrin I is that tetracarboxylic porphyrin most commonly used as marker, and at the same time it is the slowest-moving of the coproporphyrin isomers. The R_F values found are such that it would be by no means easy, in the case of an unknown porphyrin of R_F indicating, for example, something between 3 and 4 carboxylic groups, to decide whether it were really a slow-running isomer of a tricarboxylic porphyrin or a fast-running isomer of a tetracarboxylic (i.e. copro-) porphyrin. This adaptation of the lutidine–water methods to " isomer-analysis " is still in its early stages.

In two other methods of " isomer-analysis " on paper the porphyrin esters are chromatographed instead of the free porphyrins, and organic solvents are used without the addition of water. The solvents are distilled but not absolutely dehydrated, and the paper is not dried. These also are presumably partition chromatograms, the bound water of the cellulose providing the second phase.

In the first of these methods, introduced by Chu, Green & Chu (1951), the relationship between R_F and carboxylic groups (in this case the methyl esters) is similar in principle to that discussed above. In addition, the coproporphyrin isomers I and III are separated. For this restricted purpose the method is very useful. When all four isomers of coproporphyrin were tested by this method, however, they were found to move in pairs, the R_F of isomer I being identical with that of II, and the R_F of III identical with that of IV (Benson & Falk, unpublished work).

The second method using the esters was developed for the specific purpose of separating uroporphyrins I and III (Falk & Benson, 1953); its use for this purpose is discussed by Nicholas & Rimington (1953). It has not yet been possible to test by this method all four uroporphyrin isomers, since unequivocal isomers II and IV are not yet available.

Berlingozzi & Serchi (1952) have also claimed separation of copro- and uro-porphyrin isomers, as the esters, on filter-paper disks. Their fixed phase was ether, and their mobile phase was a mixture of ethanol, water and HCl.

It was hoped that scale models of porphyrin isomers (see fig. 1) might help in the choice of conditions for their separation. The most obvious correlation is the much greater degree of symmetry of isomers I and II than IV, which in turn is more symmetrical than III. This may explain the movement in pairs of coproporphyrin isomers I and II, and isomers III and IV, under the conditions of Chu *et al.* (1951). It may also give reason to hope that a method may eventually be found for the separation of coproporphyrin IV from the less symmetrical coproporphyrin III, though it still appears that the conditions will have to be found empirically.

It has now become important to be able to recognize the " unnatural " isomers, II and IV, of coproporphyrin and uroporphyrin, even if only for the purpose of excluding the possibility of their presence, in minor proportions, in material from natural sources. The reasons for this have been discussed more fully elsewhere (Falk, 1954). As shown above, some progress is being made with separation of the coproporphyrins, and there is a reasonable prospect of achieving the separation of all four isomers.

These refinements and complications may dismay the more clinical reader. The limited amount of analysis required for diagnostic purposes can, however, be carried out relatively easily and quickly. Porphyrins isolated from excreta can be esterified without difficulty, and a rough estimate of the proportions of the major components can be made immediately by chromatography of the ester mixture on Al_2O_3 or MgO columns (Nicholas, 1951). The nature of these components is readily confirmed by paper chromatography (Nicholas & Rimington, 1949, 1951), and if analysis for isomers I and III is considered important, this can be carried out for coproporphyrins (Chu *et al.* 1951) and for uroporphyrins (Falk & Benson, 1953).

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CHROMATOGRAPHY OF NUCLEOTIDES AND RELATED SUBSTANCES

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1 Detection of the spots
2 General chromatographic procedure
3 Solvents
References

Until the development of chromatographic methods, analytical work on nucleic acids and their derivatives was exceedingly limited in its scope. By a lucky coincidence these substances, which are so difficult to deal with by orthodox analytical methods, are almost ideally suited to chromatographic investigation, particularly by chromatography on filter-paper. This is because all the compounds which contain purine or pyrimidine rings in their structure have well-marked and characteristic ultra-violet absorption spectra, the molecular extinction coefficients being of the order of 10,000. Consequently it is possible to detect the presence of the spots and to make quantitative estimates of the amount of material present in each spot without the use of any spraying reagents, and, indeed, without destroying any of the substance at all.

Starch column chromatography has also been employed for this purpose (Reichart, 1948; Harris, Beale & Roe, 1950; Daly, Allfrey & Mirsky, 1950), but its deficiencies of slowness and relative inaccuracy are not balanced by any positive advantages.

Ion-exchange chromatography has been developed very largely by Cohn and his associates in the USA and has been used to effect some spectacular separations. It is also capable of handling much larger amounts of material than are the other methods, but it does suffer from slowness. Its main use in Great Britain has been for preparative work and in the identification of the isomeric pyrimidine nucleotides (Brown, Dekker & Todd, 1952). A review of the potentialities of the method is given by Cohn (1951).

In general for routine analysis, chromatography on paper has the advantages of simplicity, speed and accuracy, and will be described in detail.

1. Detection of the Spots

The whole success of a chromatographic method is dependent upon having a sensitive and convenient method for finding the spots. The original methods (Vischer & Chargaff, 1948) relied upon the formation of mercury complexes, which were then visualized by converting the mercury to the sulphide, or by making arbitrary cuts and estimating the quantity of material in each cut individually (Hotchkiss, 1948). The former method was cumbersome and relatively insensitive, while the latter lacked the necessary resolving power.

Shortly afterwards two different methods were described in Great Britain (Holiday & Johnson, 1949; Markham & Smith, 1949a). Both are of about the same sensitivity and one or other is invariably used nowadays. Both methods depend upon the fact that the ultra-violet absorption maxima of the various compounds lie near the intense and easily isolated mercury lines at 254 mμ. and 265 mμ.

Holiday and Johnson's method, which was also discovered independently in the USA by Carter (1950), depends upon the fact that filter-paper contains fluorescent impurities. The presence of an absorbing spot is thus seen quite easily as a dark area on a lighter background. A low-pressure mercury arc lamp (which may emit as much as 80% of its radiation at the resonance line of 254 mμ.), coupled with a quartz glass filter, is used. Such lamps with suitable filters are available commercially.

The other method is rather more laborious but provides a permanent, natural-size record of each chromatogram, advantages which may often outweigh the extra trouble involved. The chromatogram is used as a photographic negative and its image is printed on reflex document paper. The light used is highly filtered, a $CoSO_4$–$NiSO_4$ solution and Cl_2 gas being used to isolate the 254 mμ. and 265 mμ. lines from a high-pressure mercury arc lamp. (A simple source of light incorporating these filters is described by Markham & Smith, 1951.) By this means a photograph is obtained showing absorbing spots as white areas on a black background (fig. 1). The spots are extremely sharp because of the accentuation of contrast by the photographic procedure. The great sensitivity of this method permitted the detection of 5-methylcytosine as a minor constituent of deoxyribonucleic acid by Wyatt (1950).

The photographic method also lends itself to an extremely sensitive procedure for detecting spots of substances, such as guanine or its derivatives, which fluoresce under certain circumstances (Markham & Smith, 1950), and this method has been used to detect the presence of small amounts of 8-azaguanylic acid in various specimens of ribonucleic acid from

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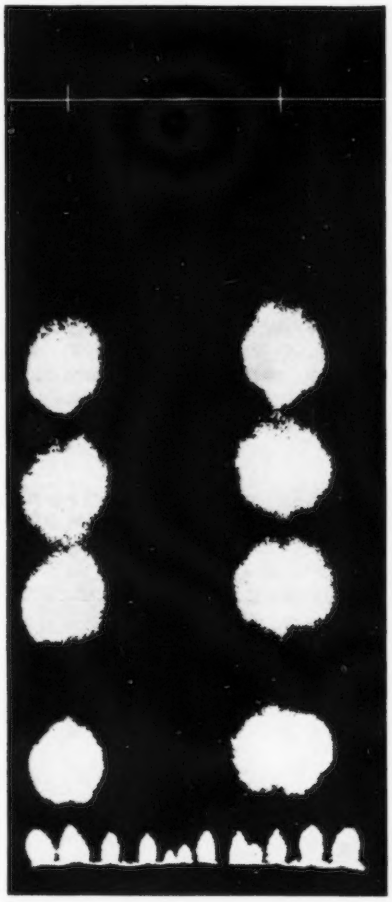
FIG. 1. A HYDROLYSATE OF YEAST RIBONUCLEIC ACID RUN IN SOLVENT 4



Total distance run: 23 cm.

Substances in order of increasing R_F: guanine (71 μg.), adenine (75 μg.), cytidylic acid (137 μg.), uridylic acid (148 μg.)

organisms treated with the purine analogue, 8-azaguanine (Matthews, 1953; Lasnitzki, Matthews & Smith, 1954). To obtain records of fluorescent spots, the short-wave ultra-violet light passing through the filter-paper is prevented from reaching the photographic paper by a thin nitrocellulose film sandwiched in between them. The sensitivity is limited only by the fluorescence of the filter-paper.

Other detection methods which are rather more limited in their scope are the various phosphorus sprays such as that of Hanes & Isherwood (1949), which has been used by Boulanger & Montreuil (1951) in Belgium. Hydrolysis of the phosphate esters may be accomplished by heat or by light, or even by enzymic methods (Fletcher & Malpress, 1953). It should be noted that nucleoside diphosphates are extremely resistant to attack by phosphatases. A method which does not rely upon hydrolysis has been published by Wade & Morgan (1953). This depends upon the fixing of ferric ions by phosphates, excess being detected by spraying with sulphosalicylic acid.

The various sugar groups, particularly those associated with purines, have been detected by methods described by Buchanan, Dekker & Long (1950). Ribonucleosides and their 5′-phosphates are oxidized by $NaIO_3$ at $C_{(2')}$ and $C_{(3')}$ to give dialdehydes. These may be detected by Schiff's reagent, as can purine deoxyribosides after acid hydrolysis. Bial's reaction for ribose and the Dische method for deoxyribose may also be used.

2. General Chromatographic Procedure

Descending chromatography is generally used so that the solvent may be run off the paper, and small glass tanks are preferable. Whatman No. 1 paper is suited to general use, and No. 3 MM may be used for preparative work, in which case the material is best applied as a continuous line. For quantitative analysis about 50 μg. of each base or its equivalent should be present in each spot, though smaller amounts may be used if necessary. Spots of 10–15 μl. are applied. Smaller spots have no advantage as they always increase in size. Blanks are taken from parallel lanes at the same level as are the experimental spots, and both are extracted in 5 ml. of 0.1 N-HCl overnight before the extinctions are read. The blanks should not exceed 0.05 in density at the bottom of the chromatogram and at the top should be 0.01–0.02. The density of extracts from the experimental spots should lie in the range 0.5–1.5 and triplicates should agree within 1% if accurate micropipettes are used.

3. Solvents

The choice of solvent is somewhat restricted by the fact that substances that absorb ultra-violet light cannot be used. Most systems which have been developed are based on the aliphatic alcohols, which may be used in a neutral, acid or alkaline state. In general the most important factor is the relative amount of water in the solvent, and many systems used may be replaced by mixtures based on miscible alcohols such as *iso*propanol. The solvents listed are relatively insensitive to changes in temperature or composition, and do not require washed papers or equilibration of the paper with the vapour. In all cases the same solution should be used to saturate the tank atmosphere.

A list of solvent mixtures which may be used to effect almost any required separation is given below. In some cases the composition is not exactly that given in the reference.

Solvent 1. *n*-Butanol (860 ml.), water (140 ml.) (Hotchkiss, 1948; Markham & Smith, 1949b).

Solvent 2. As solvent 1 but with an ammonia atmosphere (5% by volume NH_3 solution, sp. gr. 0.880) added to the liquid in the bottom of the tank. Various minor modifications of this have been used by a number of workers.

Solvent 3. *n*-Butanol (770 ml.), water (130 ml.), 98–100% formic acid (100 ml.).

R_F values of various materials in these solvents are given in Table I (Markham & Smith, 1949b; Wyatt, 1954). Adequate marker substances should be used, especially with solvent 2, which is not suitable for quantitative work with guanine or uric acid; these are best run in solvents 5 or 3 respectively (Leone & Scala, 1950). Solvent 3 is particularly useful for nucleosides. In all three solvents nucleotides remain near the origin.

TABLE I. THE R_F VALUES OF BASES AND NUCLEOSIDES IN SOLVENTS BASED UPON *n*-BUTANOL

(A certain amount of variation is to be expected with changes in temperature, etc. The values for the methylated cytosine derivatives are taken from Wyatt, 1954.)

	Solvent 1	Solvent 2	Solvent 3
Adenine	0.38	0.28	0.33
Guanine	0.15	0.11	0.13
Hypoxanthine	0.26	0.12	0.30
Xanthine	0.18	0.05	0.24
Uracil	0.31	0.19	0.39
Thymine	0.52	0.35	0.56
Cytosine	0.22	0.24	0.26
5-Methylcytosine	0.29	0.27	—
5-Hydroxymethylcytosine	0.13	0.12	—
Uric acid	—	—	0.14
Adenosine	0.20	0.22	0.12
Guanosine	0.15	0.03	0.17
Inosine	—	0.03	—
Uridine	0.17	0.08	0.25
Cytidine	0.12	0.11	0.18

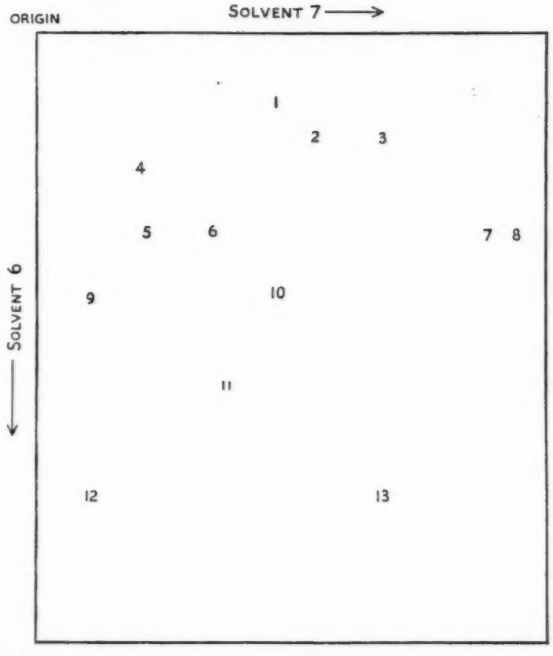
Solvent 4. *tert.*-Butanol (700 ml., at 26° C.), constant boiling HCl (132 ml.), water to 1 l. at 20° C. (Smith & Markham, 1950). This mixture has been used for the analysis of ribonucleic acids (Markham & Smith, 1950, 1951; Knight, 1953; Hamoir, 1952; Whitfeld, 1953). It separates guanine, adenine, cytidylic acid and uridylic acid with R_F values of about 0.21, 0.32, 0.48 and 0.79 respectively (fig. 1).

Solvent 5. *iso*Propanol (680 ml.), concentrated HCl (176 ml.), water to 1 l. (Wyatt, 1951). This solvent will separate the mixture resolved by the previous solvent, but with R_F values of 0.27, 0.40, 0.61 and 0.79, the order being the same. It will also resolve the bases from any known nucleic acid, the other R_F values being cytosine (and 5-hydroxymethylcytosine), 0.54; 5-methylcytosine, 0.60; uracil, 0.73; and thymine, 0.86. A run of about 30 cm. is needed for complete separation. This solvent and the previous one will move any required quantity of any base, even guanine, without tailing. The HCl must be removed thoroughly and without the use of heat, which would char the paper. Spots of HCl—or even 50% (w/v) $HClO_4$—containing hydrolysates need not be neutralized, or even dried, before the chromatograms are run. This solvent has been used by a number of workers for the analysis of deoxyribonucleic acid and for routine analysis of mononucleotides and small polynucleotides (Smith & Stoker, 1951; Smith & Wyatt, 1951; Wyatt, 1951, 1952; Elmes, Smith & White, 1952; Markham & Smith, 1952a, 1952b, 1952c; Brown, Dekker & Todd, 1952; Smith & Markham, 1952a, 1952b; Wyatt & Cohen, 1952a, 1952b; Schramm & von Kerekjarto, 1952; Sherratt & Thomas, 1953). Somewhat similar mixtures have been used by Laland, Overend & Webb (1952), and Woodhouse (1954), for the analysis of deoxyribonucleic acids.

Solvent 6. *iso*Propanol (700 ml.), water (30 ml.) + 0.35 ml. NH_3 solution (0.880 sp. gr.) per litre of tank volume (Markham & Smith, 1952a). This mixture separates the small ribopolynucleotides and mononucleotides, both in the 2′- or 3′-form and in the cyclic 2′:3′-form, and has also been used for the separation of deoxyribodinucleotides and dinucleoside monophosphates (Smith & Markham, 1952a, 1952b). It is also useful for the separation of cyclic mononucleotides (Brown, Magrath & Todd, 1952) and esters of nucleotides (Brown, Heppel & Hilmoe, 1954). Some of the separations possible with this as one dimension of a two-dimensional system are shown in figs. 2 and 3, which deal with the mononucleotides and dinucleotides found in ribonuclease digests.

Solvent 7. Saturated $(NH_4)_2SO_4$ in water (800 ml.), 1 M-Na acetate (200 ml.), *iso*propanol (20 ml.) (Markham & Smith, 1951). This mixture separates ribonucleoside 3′-phosphates from the 2′-phosphates if the base has an amino group. It also separates the 2′:3′-phosphates from the 2′- or 3′-phosphates, and will also resolve esters of isomeric adenylic, guanylic and cytidylic acids (Merrifield & Woolley, 1952; Brown *et al.* 1954; Whitfeld & Markham, 1953; Heppel, Markham & Hilmoe, 1953; Matthews, 1953; Markham & Smith, 1952a, 1952b, 1952c). It will also separate adenosine, adenosine 5′-phosphate, adenosine diphosphate and adenosine triphosphate in that order.

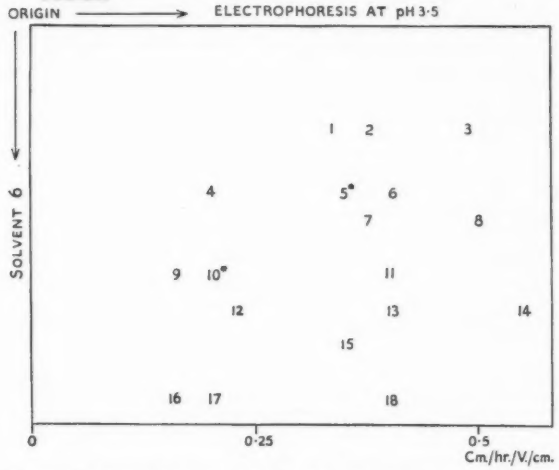
FIG. 2. POSITIONS OF MONONUCLEOTIDES AND DINUCLEOTIDES WHEN RUN IN SOLVENT 6, FOLLOWED BY SOLVENT 7



Substances separated are: 1: GC + GU; 2: G*b*; 3: G*a*; 4: AC + AU; 5: A*b*; 6: A*a*; 7: C*b*; 8: C*a* + U*a* + U*b*; 9: AC! + AU!; 10: CC! + CU!; 11: G!; 12: A!; 13: C! + U!

A, G, C and U refer to adenylic acid, guanylic acid, cytidylic acid and uridylic acid respectively. Dinucleotides are indicated by the appropriate two letters. *a* and *b* denote terminal 2′- and 3′-phosphate groupings; an exclamation mark (!) denotes a cyclic 2′:3′-phosphate termination. All the non-cyclic dinucleotides shown have a 3′-phosphate termination.

FIG. 3. SEPARATION GIVEN BY SOLVENT 6, FOLLOWED BY ELECTROPHORESIS AT pH 3.5 ON PAPER



Substances shown are: 1: GC; 2: AG; 3: GU; 4: AC; 5*: G; 6: AU; 7: GC!; 8: GU!; 9: C; 10*: A; 11: U; 12: AC!; 13: AU!; 14: UU!; 15: G!; 16: C!; 17: A!; 18: U!

Conventions are as for fig. 2.

A(10*) and G(5*) are used as markers in the chromatography. This system does not give complete resolution of the isomers having 2′- and 3′-phosphate terminations.

A similar mixture but one having rather less resolving power is given by Carter (1950). This is, effectively, 5% Na_2HPO_4 in water, saturated with *iso*amyl alcohol. It has been used for the analysis of the purine nucleotides by Brown, Magrath & Todd (1952) and by Brown & Todd (1952).

Other mixtures of unusual composition include *iso*butyric acid-ammonium *iso*butyrate (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950), which separates guanylic acid plus uridylic acid from cytidylic and adenylic acids, and a mixture of phenol–*tert.*-butanol–formic acid, which separates the four mononucleotides from ribonucleic acid (Boulanger & Montreuil, 1951). Unfortunately the latter solvent absorbs ultraviolet light.

An interesting system has been used by Eggleston & Hems (1952) for the separation of adenosine triphosphate, adenosine diphosphate and adenylic acid, in that order. Two solvents are run in succession, first *iso*propyl ether (90 ml.), 90% formic acid (60 ml.), used ascending, followed by a descending run with *n*-propanol (240 ml.), NH_3 solution, sp. gr. 0.880 (120 ml.), 0.02-M-ethylenediaminetetraacetic acid (40 ml.).

Water alone has been used (Markham & Smith, 1949b; Zamenhof, Brawerman & Chargaff, 1952).

Advantage has also been taken of the tendency of ribonucleosides and their 5′-phosphates to form borate complexes and thus to move more slowly in aqueous ethanolic solvents (Rose & Schweigert, 1951). This has also been applied to ion-exchange chromatography and paper electrophoresis (Jaenicke & von Dahl, 1952; Jaenicke & Vollbrechtshausen, 1952).

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THYROID HORMONES

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1 Advances in thyroid physiology through the application of chromatography
a Thyroidal iodine
b Blood iodine
c Tissue iodine and thyroid hormone metabolites
d Miscellaneous applications
2 Methods
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b Column chromatography
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The earliest investigations in the chemistry of the thyroid showed that the active principle of the thyroid gland was probably an organic iodine compound. Thus, in 1896, Baumann demonstrated such a substance (iodothyrin) in hydrolysates of the gland. Later in the same year Baumann & Roos (1896) showed that virtually all of the thyroidal iodine was to be found in a protein fraction extractable from the gland with dilute sodium chloride. However, the proportion of iodine present was so low that isolation of an active constituent was rather difficult. It was not until 1915 that Kendall was able to isolate, from thyroid, crystalline thyroxine, which was then characterized and synthesized by Harington (1926; Harington & Barger, 1927). This was followed by the identification and synthesis of diiodotyrosine in the thyroid gland (Harington & Randall, 1929; Foster, 1929).

From these results it was clear that the greater portion of the iodine present in the thyroid was incorporated into a protein, thyroglobulin, in the form of thyroxine and diiodotyrosine, and that this protein contained virtually all of the physiological activity of the gland. In addition to thyroglobulin, a small and variable fraction containing inorganic iodide was also detected. This basic pattern remained virtually unchanged until radioactive iodine[1] and chromatographic methods became available.

1. Advances in Thyroid Physiology through the Application of Chromatography

The use of radioactive iodine in the study of the physiology of the thyroid gland permitted analyses to be carried out on an ultra-microscale. It became apparent that in addition to the two iodine fractions of the thyroid originally described (i.e., thyroglobulin and iodide) there was a third non-thyroglobulin iodine fraction which contained some small iodine-containing molecules (Leblond & Gross, 1949).

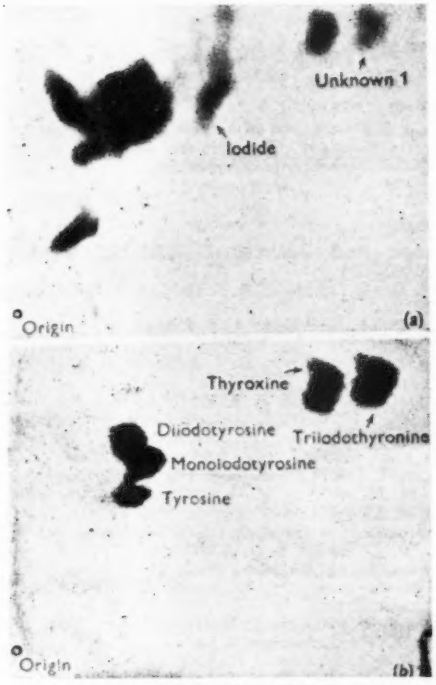
Paper chromatography was then used to characterize these fractions in the thyroid, and to analyse the extra thyroidal iodine present in the circulation or in the organs. In addition paper chromatography was used as an easy method for the purification of thyroid substances synthesized with radioactive iodine (Lemmon, Tarpey & Scott, 1950). The technique was then applied to analyses of the metabolites of these labelled preparations after injection into the body. The results obtained are briefly outlined in the following paragraphs.

a. Thyroidal Iodine

i. *Thyroglobulin iodine.* Fink, Dent & Fink (1947) analysed hydrolysates of thyroid, from rats given ^{131}I, on two-dimensional paper chromatograms. Iodide, diiodotyrosine and thyroxine were detected, together with several unidentified iodine-containing compounds.

One of the latter was then identified as monoiodotyrosine (Fink & Fink, 1948; Tishkoff, Bennett, Bennett & Miller,

FIG. 1. AUTORADIOGRAPH OF PAPER CHROMATOGRAM, AND PAPER CHROMATOGRAM ITSELF, OF A THYROGLOBULIN HYDROLYSATE



Gross & Pitt-Rivers (1953) by permission of Biochemical Journal

a: autoradiograph of paper chromatogram of a hydrolysate of rat thyroglobulin labelled with radioactive iodine

b: the paper chromatogram itself, showing the location of known iodinated compounds added with the radioactive material

The developing solvents were butanol–acetic acid vertically and butanol–dioxan–ammonia horizontally. The paper had been sprayed with diazotized sulphanilic acid. The apparent lack of separation of monoiodotyrosine and diiodotyrosine in the autoradiograph is due to the long exposure required to visualize the much weaker triiodothyronine image. This resulted in over-exposure of the more strongly radioactive substances, with a resultant spreading of the photographic reaction.

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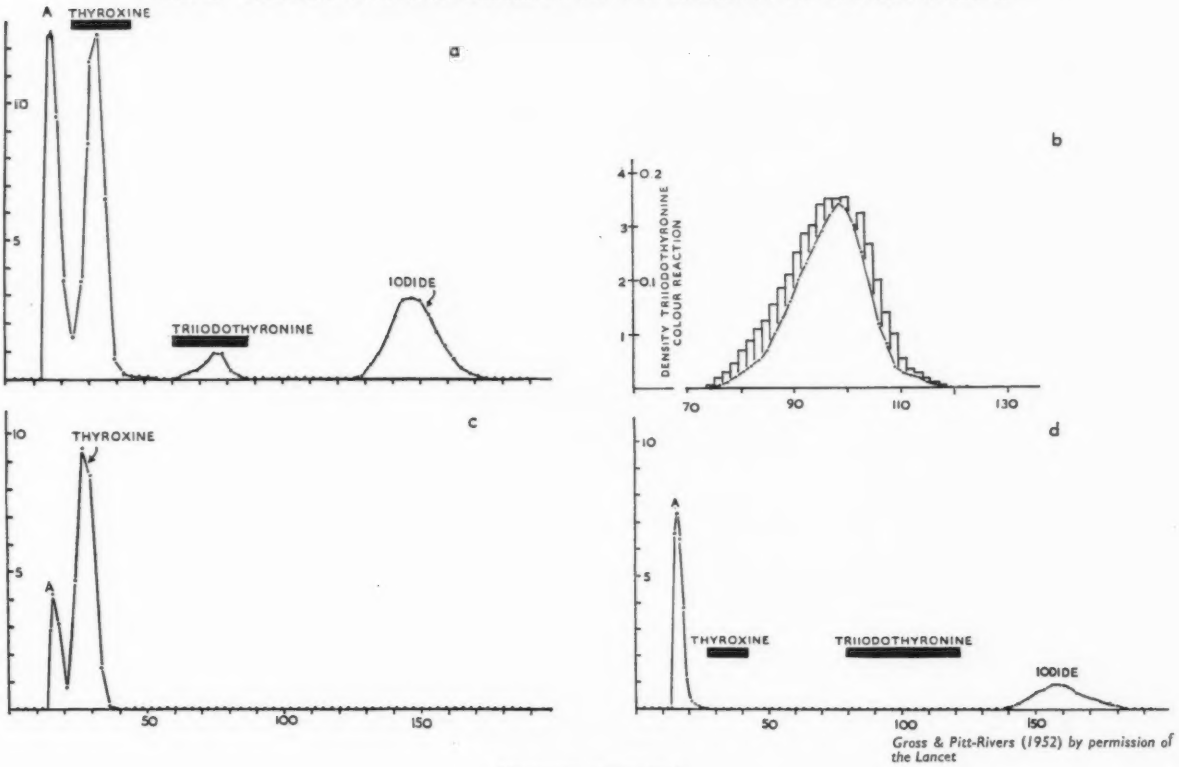
[1] See Gross & Pitt-Rivers, *Brit. med. Bull.* 1952, 8, 136.—ED.

FIG. 2. COLUMN CHROMATOGRAPHY IN THE ANALYSIS OF HUMAN PLASMA



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Abscissae: eluate (ml.)
Ordinates: counts/sec.

a: analysis on a kieselguhr column of the plasma radioactivity from a patient (diagnosis: carcinoma, hypothyroid), 24 hr. after he had been given 100 mC radioiodine; 3 mg. of non-radioactive thyroxine and triiodothyronine were added to the plasma sample before the analysis.
The curves represent the distribution of radioactivity in successive fractions of the effluent from the column.
The horizontal black bars indicate the positions of the added thyroxine and triiodothyronine determined colorimetrically.
A is an artifact.

b: the triiodothyronine peak from a column analysis of radioactive human plasma from a patient (diagnosis: hyperthyroidism), 24 hr. after he had been given 4 mC radioiodine. Figure shows the correspondence between the distribution of radioactivity (broken line) and the distribution of added triiodothyronine determined colorimetrically (shown as blocks).

c: column analysis of non-radioactive human plasma to which radioactive thyroxine has been added before extraction. It is noted that no radioactive triiodothyronine or iodide was detected; however, a radioactive substance (A) appeared at the front of the column.

d: column analysis of non-radioactive human plasma to which $Na^{131}I$ had been added together with 2 mg. each of thyroxine and triiodothyronine. It is noted that no radioactive thyroxine or triiodothyronine was detected; this indicates that no exchange has taken place (see text, p. 223). The peak (A) of radioactivity at the front is derived from iodide and probably represents an artifact caused by the procedure.

1949). By quantitative assessment of their chromatographic separation, Taurog, Tong & Chaikoff (1950) showed that monoiodotyrosine may constitute as much as 15% of the total thyroglobulin iodine. Similarly Roche, Jutisz, Lissitzky & Michel (1951) quantitatively analysed both baryta and tryptic digests of thyroglobulin and were able to demonstrate that monoiodotyrosine was not an artifact of alkaline hydrolysis despite the decomposition of a large part of the diiodotyrosine.

The same authors have also identified small amounts of monoiodohistidine and traces of diiodothyronine in thyroglobulin, using two-dimensional chromatograms (Roche, Lissitzky & Michel, 1951). A third amino acid, triiodothyronine, was identified on chromatograms of thyroid hydrolysates (fig. 1) (Gross & Pitt-Rivers, 1953; Roche, Lissitzky & Michel, 1953b) and was isolated and purified on a kieselguhr column (Gross & Pitt-Rivers, 1953).

ii. *Non-thyroglobulin iodine.* It had been previously shown that thyroglobulin itself was not secreted by the thyroid into the circulation, indicating the possible existence of smaller iodine-containing compounds in the thyroid (i.e. non-thyroglobulin iodine). Such a fraction was analysed by paper chromatography; it was found to contain thyroxine (Gross, Leblond, Franklin & Quastel, 1950; Gross & Leblond, 1951a; Tong, Taurog & Chaikoff, 1951), monoiodotyrosine and diiodotyrosine (Tishkoff *et al.* 1949; Gross *et al.* 1950; Roche, Deltour, Lissitzky & Michel, 1950; Gross & Leblond, 1951a; Tong *et al.* 1951). In addition a substance occurring

TABLE I. RELATIVE MOVEMENT (R_F) OF BIOLOGICALLY OCCURRING COMPOUNDS ON PAPER CHROMATOGRAMS WITH VARIOUS SOLVENTS

(The upper layer is taken for solvent, and the substances in parentheses indicate what the solvent was equilibrated with in each case.)

Compound	*n*-Butanol (2 N-acetic acid)	*n*-Butanol (2 N-formic acid)	*n*-Butanol (4 vol.), dioxan (1 vol.) (2 N-NH_4OH)	*n*-Butanol (2 N-NH_4OH)	Collidine (conc. NH_4OH)	*iso*Pentanol (6 N-NH_4OH)	*n*-Butanol (1 vol.), pentanol (1 vol.) (2 N-NH_4OH)	Collidine (1 vol.), lutidine (1 vol.) (water)
Thyroxine	0.88	0.91	0.45	0.58	0.45	0.15	0.41	0.79
Triiodothyronine	0.91	0.91	0.65	—	0.65	0.27	—	—
Diiodothyronine	0.85	0.85	0.60	0.73	—	0.38	0.63	—
Thyronine	—	—	—	—	—	—	0.28	—
Diiodotyrosine	0.70	0.69	0.11	0.08	0.11	—	0.20	0.56
Monoiodotyrosine	0.57	0.51	0.17	0.13	0.28	—	0.20	0.68
Tyrosine	0.50	—	0.12	—	0.25	—	0.20	—
Iodide	0.27	0.29	0.40	0.37	0.90	0.08	—	0.89
Iodine	1.00	1.00	0.40	—	—	—	—	—
Thyroxine "glucuronide"	0.45	—	0.33	—	0.20	—	—	—
Triiodothyronine "glucuronide"	—	—	—	—	0.28	—	—	—
Monoiodohistidine	0.10	—	—	0.21	—	—	—	—
Diiodohistidine	—	—	—	0.13	—	—	—	—

in the same location as triiodothyronine, as well as some other unknown substances, were demonstrated (Gross *et al.* 1950; Gross & Leblond, 1951a).

b. Blood Iodine

The nature of the circulating thyroid hormone had been a matter for speculation and discussion. Most of the evidence favoured the view that thyroxine was the predominant constituent of the blood iodine. This was clearly established by the use of paper chromatography in the analysis of animal and human plasma (Laidlaw, 1949; Taurog, Chaikoff & Tong, 1950; Gross *et al.* 1950; Rall, 1950; Rosenberg, 1951; Gross & Pitt-Rivers, 1951), and was further confirmed by the use of column chromatography (fig. 2) (Gross & Pitt-Rivers, 1952). In addition the presence of triiodothyronine in the circulation was demonstrated both by the paper and column methods (Gross & Pitt-Rivers, 1952).

c. Tissue Iodine and Thyroid Hormone Metabolites

Studies on the nature of the tissue iodine are still in their preliminary stages. After the injection of thyroxine into rodents, paper chromatographic analysis was carried out on the ^{131}I of the organs and excreta (Gross & Leblond, 1951b). The liver, kidney and faeces were found to contain thyroxine and a substance similar in behaviour to triiodothyronine. The kidney and liver contained in addition an unknown substance which was later shown to be a readily hydrolysable conjugate of thyroxine (Leblond & Cambron, 1952). The urine radioactivity was predominantly in the form of iodide. Similar experiments on the bile of thyroxine-treated rats indicated the presence of a thyroxine conjugate which was readily hydrolysed with β-glucuronidase (Taurog, Briggs & Chaikoff, 1952). A similar triiodothyronine conjugate was detected on paper chromatograms of the bile from rats treated with radioactive triiodothyronine. This conjugate contained most of the biliary iodine, with the remainder being distributed between free triiodothyronine and an unidentified iodinated compound (Roche, Michel & Tata, 1953).

Lachaze & Thibault (1951) have presented chromatographic evidence which they believe to indicate the conversion of thyroxine to thyroxamine in the intestinal mucosa.

It is clear that this summary represents only the initial phases of the investigations into the biosynthesis and metabolism of the thyroid hormone and that these have been made possible by the development of chromatographic methods of analysis.

d. Miscellaneous Applications

Chromatography has also been useful as a purification procedure in the preparation of ^{131}I-labelled thyroxine and other of the iodinated substances occurring in the thyroid (Lemmon *et al.* 1950; Gross & Leblond, 1951b). It has also been used to follow the stages in the iodination of tyrosine (Roche, Lissitzky, Michel & Michel, 1951a), histidine (Roche, Lissitzky, Michel & Michel, 1951b) and diiodothyronine (Roche, Lissitzky & Michel, 1953a) in the course of the formation of the monoiodo- and diiodo-substituted derivatives.

2. Methods

a. Paper Chromatography

Most analyses in thyroid hormone biochemistry have been carried out with paper partition chromatography. Whatman No. 1 paper has been used almost exclusively by either the ascending or descending methods.

For the most part separations have been carried out on a mixture labelled with radioactive iodine, since the components occur in such minute concentrations in the body that they are not chemically detectable in the amounts of extract that are separable on paper. In order to identify the radioactive components, chemically detectable amounts of pure iodinated compounds (e.g., thyroxine, diiodotyrosine, etc.) are chromatographed together with the radioactive mixture. Identification of a labelled substance depends on the correspondence of its location with that of one of the known substances (cf. fig. 1). Little dependence is placed on these R_F values,

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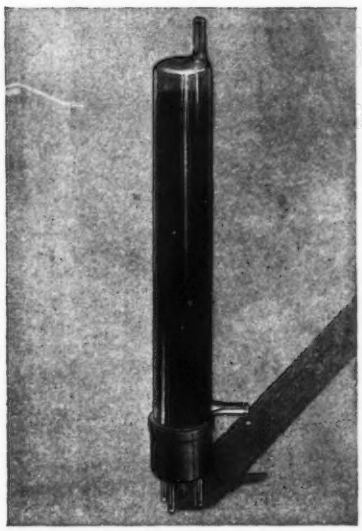

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M. 6; M. 6H	Blood volume measurements, using ^{32}P and all liquid counting applications
G. 10	Blood circulation investigations

TYPE	APPLICATIONS
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EW. 3H	Fat synthesis from small molecules (^{14}C)
G. 10H ; B. 12H	Health monitoring and precautions in handling radioactive isotopes
N1b	Bone Surgery
N1K	Brain Surgery

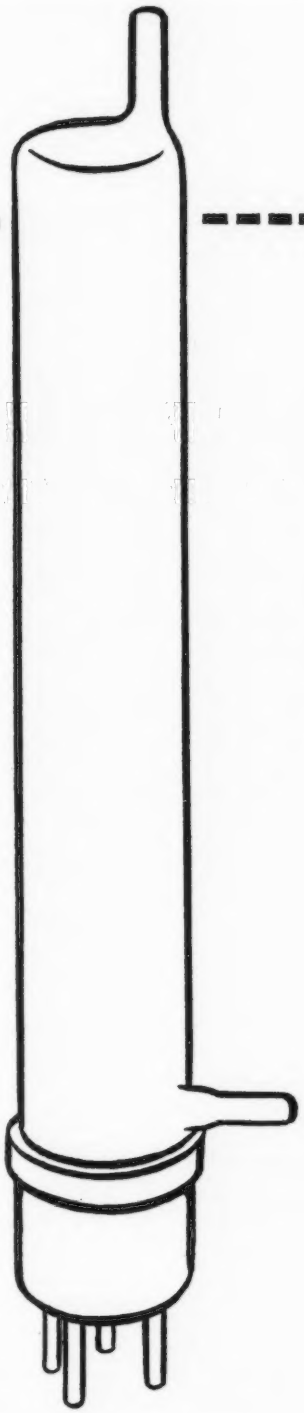

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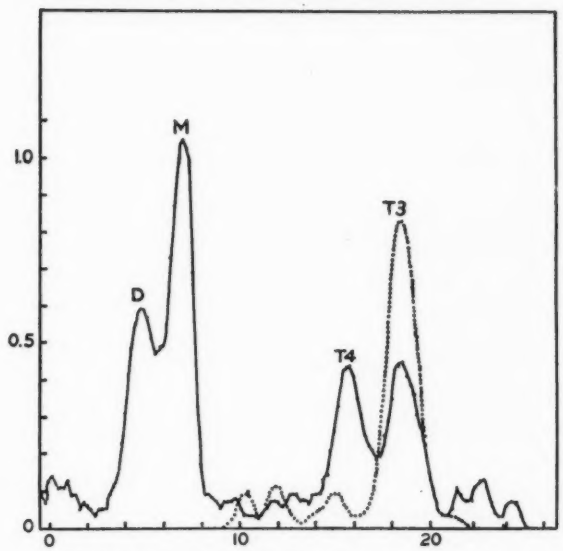
FIG. 3. TYPICAL GRAPH OBTAINED WHEN INTENSITIES OF SPOTS ON CHROMATOGRAMS AND ON THE CORRESPONDING AUTORADIOGRAPHS ARE MEASURED WITH A DENSITOMETER



Abscissae: cm. along paper strip chromatogram
Ordinates: units of optical density
——— spots on chromatograms
..... spots on autoradiographs

An extract of a tissue incubated with radioactive triiodothyronine (T3) was fractionated one-dimensionally with butanol–dioxan–ammonia, together with non-radioactive diiodotyrosine (D), monoiodotyrosine (M), thyroxine (T4) and triiodothyronine (T3) as carriers. After the autoradiograph was obtained, the carriers were visualized with diazotized sulphanilic acid. The intensity of colour was measured with a green filter along a band 5 mm. wide running through the centres of the spots. Using a slit 1 mm. wide, the density of every alternate millimetre of band was measured. Exactly the same area of the autoradiograph was measured without using a filter.
This type of evaluation establishes clearly the correspondence (e.g., in the case of T3) or lack of correspondence (the small peak in the region of T4) of the radioactive peaks with carrier peaks. In addition the area under the curves gives an estimate of the relative distribution of the radioactivity detected by the autoradiograph.

since the amounts of extraneous material in the radioactive mixture may heavily load the paper (especially in those cases where the radioactivity content is low) and thereby depress the migration of the various substances. With this consideration in mind, Table I has been prepared from the data in the literature. The values are given only as an indication of the relative movements of those biologically occurring iodinated substances in the various solvents.

It has been the experience of several of the investigators in this field that chromatography in at least two dimensions is essential for identification. This became especially clear to the writer when, in association with Mrs R. Pitt-Rivers, he was carrying out work on the identification of triiodothyronine. In a preliminary screening of a number of thyroxine analogues and peptides synthesized by Sir Charles Harington and Mrs Pitt-Rivers, several test substances migrated in a very similar fashion to the—then unidentified—radioactive spot. However, on chromatographing with a different solvent pair, the lack of identity was demonstrated. The ultimate identification in this case was based not only on two-dimensional chromatography with several solvent pairs but also on isolation of the unknown substance on a chromatographic column (see section 2b).

The methods for locating substances on the paper may be divided into (i) those used to detect the various radioactive components of mixtures and (ii) those used to visualize the added known non-radioactive substances.

The qualitative distribution of radioactivity on the chromatograms is most readily carried out by applying photographic film to the paper; after a suitable period of exposure, the film is developed and the black areas (i.e. autoradiographs) indicate the location of the radioactive spots. In practice, the chromatogram is applied to a sheet of Eastman No-Screen *x*-ray film with a sheet of cellophan intervening between the two. The resultant sandwich is stapled together in several places and inserted into an *x*-ray cassette or industrial film holder for exposure. The cellophan membrane is used to avoid the possibility of chemical reduction of the photographic emulsion by substances on the chromatogram. The stapling not only acts to keep film and paper in close contact, but after development the resulting holes are used to register accurately the autoradiograph with the chromatogram.

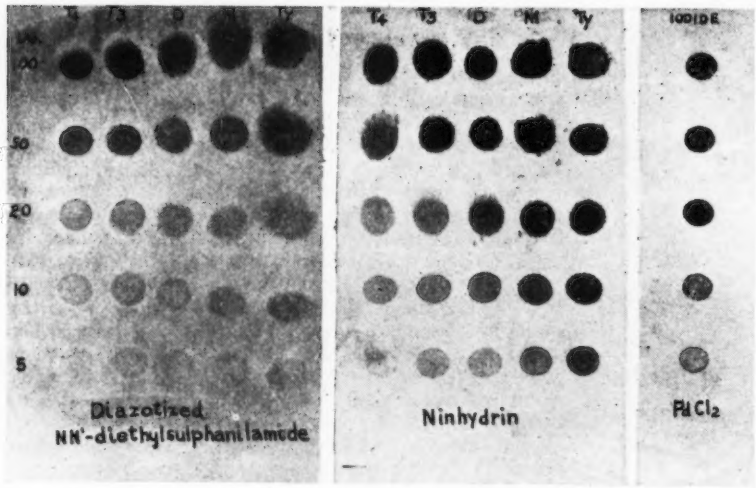
Quantitative estimations have been carried out on one-dimensional chromatograms by either cutting out the area corresponding to a particular substance and determining its radioactivity, or by counting successive segments of the paper from origin to solvent front under a Geiger-Müller counter (cf. Lissitzky, 1952). The latter method gives a more accurate distribution profile but is rather tedious unless an automatic scanning device is used. Such instruments are relatively easily constructed and are available commercially. An attempt has been made to assess quantitatively autoradiographs of such chromatograms. This is carried out by measuring the densities of successive portions of the film with a densitometer and plotting the values obtained. If Eastman No-Screen *x*-ray film is used, the densities obtained are linearly related, within limits, to the amount of incident β radiation (Yagoda, 1949). The paper itself can be similarly scanned after its carriers have been visualized by spraying. The results of such a plot on film and paper are shown in fig. 3.

This procedure has the following theoretical advantages. Firstly, during the course of exposure there is an integration of the emitted radiation over the useful life of the isotope. Secondly, the densitometric procedure is relatively rapid and highly sensitive. This permits narrower segments of films to be measured, resulting in a more accurate profile with better resolution between closely adjacent substances.

A possible source of error in these quantitative methods lies in the variation in the absorption of β particles by the paper, due to its variation in density. The error introduced is probably not large, since Winteringham, Harrison & Bridges (1952) showed that, while a paper of 8.79 mg./cm.^{2} density absorbs about 17% of the β radiation of ^{131}I, a variation of 10% in paper density causes only a 2% variation in count.

In the detection of the various iodinated substances on the paper chromatogram, ninhydrin was the first reagent to be used. The sensitivity of this reaction when carried out in the usual fashion was found to be rather low (Hird & Trikojus, 1948; Gross *et al.* 1950). In an attempt to obtain a more sensitive test, a modification of the Pauly reaction, involving

FIG. 4. A COMPARISON OF THE SENSITIVITY OF DIAZOTIZED *N'N'*-DIETHYLSULPHANILAMIDE AND NINHYDRIN IN THE DETECTION ON PAPER OF THYROXINE (T4), TRIIODOTHYRONINE (T3), DIIODOTYROSINE (D), MONOIODOTYROSINE (M) AND TYROSINE (Ty)



Each spot was made by adding to the paper 10 μl. of an acid–ethanol solution containing the quantities shown in left-hand column. After development the papers were photographed by transmitted light. The ninhydrin reaction resulted in the lowest background colour, showed the highest sensitivity for all the substances tested, and the least diffusion of the spots.
The sensitivity of $PdCl_2$ as a spot test for iodide is shown in right-hand photograph.

coupling with diazotized sulphanilic acid, was used (Gross & Leblond, 1951a). Variants of this basic procedure have been described and in addition the use of diazotized *N'N'*-diethylsulphanilamide (Winikoff & Trikojus, 1948) as the coupling agent has been suggested (Pitt-Rivers, personal communication). The colours obtained are purple for the compounds containing the diphenolic ethers (e.g., thyroxine) and orange for the compounds containing a single benzene ring (e.g., diiodotyrosine).

Since other workers, notably Michel and his collaborators, were able to obtain a reasonable sensitivity with the ninhydrin reaction, it was felt that a systematic evaluation of the two methods might provide useful data for this review. Sheets were prepared on which were spotted in decreasing concentration 10 μl. of an acid–ethanol solution of five of the more commonly found substances, papers were sprayed with diazotized sulphanilic acid, or diazotized *N'N'*-diethylsulphanilamide (prepared as described by Gross & Leblond (1951a), except that the alkaline spray was 10% (w/v) aqueous Na_2CO_3). A third paper in the series was sprayed with 0.2% ninhydrin in *n*-butanol containing 5% acetic acid (cf. Lissitzky, 1952). When the ninhydrin-sprayed sheet was not exposed to heat but left at room temperature overnight, a stronger reaction was obtained. The results of this experiment are shown in fig. 4. The diazotized sulphanilic acid reagent was the least sensitive of the three and showed the highest background density. As may be seen from fig. 4, the ninhydrin reaction was somewhat more sensitive than that given by the diazonium reagent and showed the lowest background density. Mixtures containing 20–50 μg. of the five substances, when separated with butanol–dioxan–ammonia, were easily detected with ninhydrin.

Both reagents are useful. The diazotized diethylsulphanilamide reagent will visualize only the phenolic substances. However, it has a somewhat lower sensitivity, will cause some loss of iodine during coupling (cf. Komant, 1951) and tends to cause some spreading of the spots of diiodotyrosine, monoiodotyrosine and tyrosine. The only disadvantage of ninhydrin lies in its lack of specificity. The rather crude extracts usually analysed in this work may contain amino acids and other substances which show a highly sensitive reaction with ninhydrin; this could obscure the separations. In these cases the use of the more specific reagent is indicated.

For the detection of inorganic iodide a variety of methods have been used. These included spraying with $AgNO_3$ (Laidlaw, 1949), KIO_3, acid and starch (Lissitzky, 1952) and 1% aqueous $PdCl_2$ (Gross & Leblond, 1951a). We have found the latter reaction most useful since it is sensitive, easy to apply and results in a permanent spot. The sensitivity of this reagent for iodide is shown in fig. 4.

Recently Bowden & Maclagan (1954) have reported a highly sensitive method which is based on the catalytic effect of iodide in the reduction of ceric ions. In addition to iodide, the iodine of the organic compounds affects the reaction. The maximum sensitivities reported are 0.01 μg./spot of iodide and 0.1 μg./spot of thyroxine or triiodothyronine.

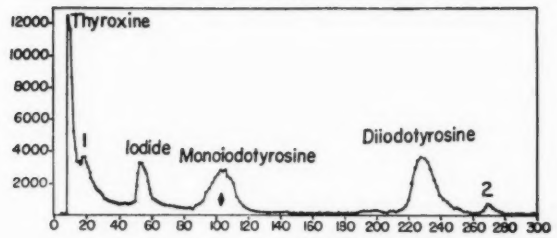
b. Column Chromatography

Because of the limited amount of material which could be separated on paper chromatograms, it became necessary to apply column chromatographic methods to some thyroid problems. For example, in the isolation of triiodothyronine from ox thyroid (Gross & Pitt-Rivers, 1953), a column was used to isolate this material from the large amount of extraneous material as well as from the thyroxine present in the preparations of the glands. The column consisted of kieselguhr with 0.5 N-NaOH as the stationary phase and 20% $CHCl_3$ in *n*-butanol as the mobile phase.

The procedure is a modification of that described by Grönkvist & Hellberg (1951). These authors used 40% $CHCl_3$ in *n*-butanol as the mobile phase with the same stationary phase. On repeating this work it was found that, despite adequate equilibration of the solvents, the stationary phase was rapidly stripped from the column, and separation could not be effected. At the suggestion of A. J. P. Martin, equilibration of the mobile phase was maintained by an additional amount of kieselguhr, containing 0.5 N-NaOH, being placed on the column, after the mixture to be separated had been added.

The effluent is fractionated in 2 ml. fractions on an automatic fraction collector. The radioactivity of the effluent may be measured by passing it through a flow-type Geiger-Müller tube (e.g., F. 10 of 20th Century Electronics Ltd.) attached to

FIG. 5. ANALYSIS ON A STARCH COLUMN OF A HYDROLYSATE OF RABBIT THYROGLOBULIN LABELLED WITH RADIOACTIVE IODINE



Dobyns & Barry (1953) by permission of Journal of Biological Chemistry

Abscissae: fractions (0.5 ml./fraction)
Ordinates: net counts/min.

1: unknown no. 1; 2: unknown no. 2

The first solvent used was *n*-butanol–propanol–0.05 N-Na_2CO_3 (1 : 2 : 1). At the point indicated by the black marker the solvent was changed to *n*-butanol–propanol–0.1 N-HCl (1 : 2 : 1). Both thyroxine and triiodothyronine appear in the front-running peak. The identity of unknowns nos. 1 and 2 has as yet not been determined.

a counting rate-meter with a recording milliammeter, or by counting the individual fractions in a scintillation counter of the "well" type. The iodinated compounds in the effluent can be estimated chemically by the nitrous acid reaction (Grönkvist & Hellberg, 1951; Gross & Pitt-Rivers, 1953) or the photometric ninhydrin reaction of Moore & Stein (1948; Albert & Braasch, 1953, personal communication). The type of separation obtained is illustrated in fig. 2. Thyroxine, triiodothyronine and inorganic iodide are adequately separated (fig. 2a). In this case the identification of triiodothyronine in human plasma was indicated by the close correspondence of the distribution of radioactivity with the distribution of added triiodothyronine as indicated by the colorimetric nitrous acid reaction (fig. 2b). An additional component occurring at the front is probably an artifact, since it appears when either thyroxine (fig. 2c) or iodide (fig. 2d) are added to plasma and subjected to the separation procedure.

Diiodotyrosine and monoiodotyrosine are strongly held in the stationary phase and are not easily eluted with 20% $CHCl_3$ in *n*-butanol. By arranging a continuous change in solvent composition, such that the $CHCl_3$ content is reduced from 20% to 0%, thyroxine, triiodothyronine, iodide, diiodotyrosine and monoiodotyrosine can be separated (Gross & Martin, 1952). This required a rather complicated apparatus for mixing and for the maintenance of a reasonable rate of flow. Recent preliminary work in this laboratory and in others (Albert & Braasch, personal communication) has indicated that the same result can be obtained if 20% $CHCl_3$ in *n*-butanol is replaced by 10% *iso*propanol in *n*-butanol after the iodide fraction has left the column.

Dobyns & Barry (1953) have described a starch column which separates thyroxine, iodide, monoiodotyrosine and diiodotyrosine (fig. 5). Thyroxine and triiodothyronine are not separated and appear at the front. This factor reduces the usefulness of this method of separation.

A possible source of error in both column and paper chromatography lies in the possibility of exchange between radioactive inorganic iodide and the organic iodine compounds. This occurs relatively easily in acid solvents (Gross & Leblond, 1951a; Dobyns & Barry, 1953), owing to the oxidation of the iodide to elemental iodine which is the intermediary in the exchange reaction. This has been prevented by adding a reducing agent such as thiosulphate (Dobyns & Barry, 1953). With alkaline solvents exchange does not occur. This is illustrated in fig. 2d, where radioactive iodide was chromatographed with thyroxine and triiodothyronine containing stable iodine; no radioactivity was detected in either the thyroxine or triiodothyronine fractions.

3. Summary

The methods of paper and column chromatography as applied to the study of the biochemistry of thyroid hormones have been evaluated and discussed. The role of those methods in providing data in this field, unattainable by previous methods, has been illustrated by a summary of the newer knowledge of thyroid physiology.

ACKNOWLEDGEMENTS

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ANTIBIOTICS AND VITAMINS

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1 Antibiotics
a Penicillins
b Streptomycins
c Polypeptide antibiotics
d Polymyxins
2 Vitamins
References

The history of the application of chromatography to the antibiotics is very nearly the modern history of chromatography itself. This is partly because of the intense biological, therapeutic and commercial interest in the antibiotics, leading to rapid and extensive application of any promising new technique, and partly because of the particular applicability of chromatographic methods to the problems involved, namely, the separation of closely similar, often labile, substances from mixtures. This, of course, is not a one-way process: the fertilization of advances by their application to new systems provides the stimulus for further invention. It is thus no accident that the proliferative phase in the discovery of the antibiotics should coincide with the remarkable renaissance of chromatography in the last decade. In the field of vitamins more attention has been focused on the water-soluble compounds by users of the newer techniques, although the classical methods of adsorption chromatography were employed with complete success in problems of the vitamin A and D groups. Ion-exchange chromatography has from the earliest days been employed in vitamin assay.

1. Antibiotics

a. Penicillins

Pride of place in any discussion on antibiotics must necessarily be given to the penicillins, and they provide an epitome of the chromatographic technique. The classical adsorbent—alumina standardized according to Brockmann—was employed by Abraham, Chain & Holiday (1942), and the penicillin extract was applied in ethereal solution; after development a large number of bands could be observed in visible and ultra-violet light. Alkalinity of the alumina caused inactivation of the labile antibiotic, and the substitution of acid-washed alumina—although causing little change in the appearance of the columns—diminished their destructiveness, but it was still necessary to run them in the cold.

Catch, Cook & Heilbron (1942) described an ingenious column consisting of silica gel containing precipitated barium carbonate, called by them a "chemical chromatographic column". Crude extracts containing penicillin, when placed on such a column in an organic solvent, appeared as sharp bands, biological activity being always associated with recognizable pigment bands. The bands moved down the column on being washed with solvent, but were incapable of genuine development. They have the characteristics of the bands of displacement analysis (Claesson, 1946), the several acidic materials displacing less acidic substances further down the column. The column material containing the bands was dissected out, and the penicillin eluted.

True partition chromatography (Martin & Synge, 1941) was adopted by two groups of workers. The Oxford workers took up a suggestion of Martin and chromatographed penicillin extracts on silica gel, using a phosphate buffer as the stationary phase, and percolated by an organic solvent. Independently, workers at Imperial Chemical Industries Ltd. arrived at the same solution (Levi, 1949; see also Boon, Calam, Gudgeon & Levi, 1948), and were enabled by this means to distinguish between penicillin (later named 2-pentenylpenicillin) produced by surface culture on a mainly synthetic medium and penicillin of American origin (benzylpenicillin) prepared from a medium containing corn-steep liquor. Differences between these penicillins were already suspected but chromatography provided the proof. This method was taken up generally and led to the isolation of a number of other members of the group in pure crystalline form.

The demonstration of paper chromatography by Consden, Gordon & Martin (1944) followed closely on the partition method, and the former was adopted by Goodall & Levi (1947) for the separation and quantitative determination of the penicillin mixtures in culture media. The problems involved in this application were considerable. The instability of the penicillins in acid solutions was diminished by working at low temperatures. Buffering of the paper reduced the loss due to the acid-catalysed re-arrangement. Ethyl ether proved to be a suitable solvent. To maintain a chromatographic chamber under conditions of equilibrium at constant relative humidity with such a volatile solvent was no mean feat. After development of the chromatogram and removal of the solvent, the paper was placed in contact with agar gel seeded with an organism susceptible to penicillin. After

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sufficient time for diffusion of the penicillin into the gel, the paper was removed and the plate incubated and examined for inhibition zones in the manner customary for penicillin assay.

These diverse methods for the separation, purification and assay of the penicillins have, of course, been widely extended. Martin and his colleagues have described improved methods (Albans & Baker, 1950; Baker, Dobson & Martin, 1950), using paper chromatography, designed to deal with large numbers of assays in a completely standardized manner. Randall & Martin (1949; see also James, Martin & Randall, 1951) described an automatic recording system for the separation and approximate assay of the penicillins. This involved the partition of the penicillin mixture between phosphate buffer and ethyl ether on kieselguhr, the conductivity of the effluent (indicating the emergence of penicillin bands) being recorded on a chart. The effluent was collected in small fractions by an ingenious mechanical fraction collector, each fraction being duly noted on the chart and correlated with the conductivity record.

It will not come amiss at this point to record the influence on chromatography of the introduction of fraction collectors. There have been many kinds described in the literature; one of the first and most elegant was that of Moore & Stein (1949). They enable the collection of a large number of equal fractions without attention, often overnight, and facilitate the quantitative analysis of column operation so necessary to the understanding of the mechanisms involved. It is the author's experience that a model based on that of James *et al.* (1951) (Model 2), which has been universally copied, is one of the most reliable and easily made.

Of great interest was the biosynthetic preparation of a radioactive penicillin mixture containing ^{35}S (Lester Smith, 1949). Following chromatography according to Goodall and Levi's method, the partition of the radioactivity on the paper was determined autoradiographically, and agreed well with the proportions of the penicillins estimated microbiologically.

What of the final synthesis of the penicillins? The workers who succeeded in extracting from reaction mixtures the minute amounts shown microbiologically and by enzyme inactivation to be present found it more convenient to use the allied technique of counter-current distribution.

b. Streptomycins

Published work on the streptomycins has appeared largely in American journals despite the considerable amount of work which must have been done in Great Britain. The streptomycins were found to be strongly basic and the original methods for their isolation and purification followed those applied to penicillin except for the complete acid-base reversal. Liquid–solid chromatography employing acid-washed alumina was the method employed in the first isolation of the pure material (see review by Peck, 1948). Streptomycin hydrochloride was adsorbed on the alumina to a lesser extent than the impurities present and, on washing with 80% (v/v) aqueous methanol, came through ahead of the latter. In an example given, 10 kg. of alumina were used to purify 160 g. of streptomycin. It is generally known that the antibiotic is purified on the large scale commercially by ion-exchange chromatography, but the details have not usually been revealed (cf. Coppock & Short, 1946). Ion-exchange chromatography has also been used to separate streptomycin and its analogue, mannosido-streptomycin, from fermentation broths for chemical assay (St John, Flick & Tepe, 1951).

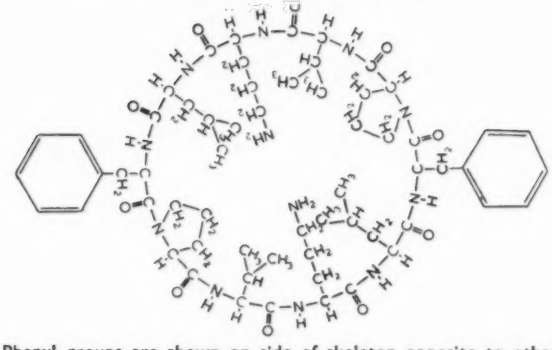
The examination by paper chromatography of fermentation broths for substances having the microbiological characteristics of streptomycin was an obvious step. The strongly basic nature of streptomycin needed an acidic solvent (Winsten & Eigen, 1948), and butanol containing *p*-toluenesulphonic acid was a solvent employed by a number of workers. An innovation in this field was the salting-out technique of Tiselius, applied by Horne & Pollard (1948). In this method, aqueous salt solutions (e.g., 0.3% ammonium chloride) were used to irrigate the paper by capillary ascent. The separated streptomycins were detected either by the Sakaguchi reaction or by the usual Goodall and Levi method of the seeded agar plate.

c. Polypeptide Antibiotics

Whereas the problems involved in the structures of the antibiotics so far mentioned have been largely solved by the ordinary methods of organic chemistry, in the investigation of polypeptide antibiotics chromatography has been indispensable. This could not have failed to have been so, since these methods were originally designed for dealing with the hitherto intractable problems of amino-acid chemistry. It was no surprise therefore that Martin and Synge and their co-workers should have interested themselves in these antibiotics. Work on the gramicidins and tyrocidine was begun by the separation and determination of the amino acids, formed by hydrolysis, as their acetamido derivatives on the silica gel partition column. We shall concentrate our attention for the moment on gramicidin S, since this is the most illustrative of the methods under discussion. By the above-mentioned method Synge (1945) showed that this contained equimolar proportions of valine, ornithine, leucine, phenylalanine and proline. Partial hydrolysis yielded a mixture from which were identified, by micromethods using paper chromatography (Consden, Gordon, Martin & Synge, 1947), the dipeptides—valyl-ornithine, ornithyl-leucine, leucyl-phenylalanine, phenylalanyl-proline—and two tripeptides (not rigorously identified)—phenylalanyl-prolyl-valine and valyl-ornithyl-leucine. A method just then introduced by Sanger (1946) enabled him to identify the only free amino group as that of the δ-amino group of ornithine; there were no free acidic groups. The method consisted in causing any free amino groups of a peptide to react with fluorodinitrobenzene to give dinitrophenyl- (DNP-) amino groups, which were identified, after hydrolysis of the peptide, by paper chromatography. The structure proposed to fit these facts is shown in fig. 1, in which the polypeptide is represented in an entirely conventional manner, the phenyl rings being supposed to show that the D-phenylalanine differs from the other L-amino acids in configuration. In agreement with this structure were the evidence for its molecular weight and, particularly, an *x*-ray analysis carried out by Crowfoot and Schmidt (see Consden *et al.* 1947). The antibiotic has been considered in this detail not because of its interest as an antibiotic, which is small, but because it is the prototype of cyclic antibiotics, and the methods employed in its structural analysis have found wide application. Similar structures have been proposed for a large number of polypeptides.

Counter-current distribution studies have shown gramicidin S to contain polypeptides other than the main component, but do not invalidate the above findings. Gramicidin was resolved into three main components and their amino-acid composition was determined by Moore and Stein (cited by Craig, 1949) by a very precise method of chromatography

FIG. 1. STRUCTURE OF GRAMICIDIN S (DIAGRAMMATIC)



Phenyl groups are shown on side of skeleton opposite to other R groups to denote opposite configuration.

(liquid gel) on starch, now superseded by their more convenient method of elution chromatography using an ion-exchange resin (Moore & Stein, 1951). Interest attaches to the gramicidins in that they were the first polypeptide antibiotics to be shown to contain a proportion of material which is not an amino acid, namely, ethanolamine. This is a common phenomenon in the polypeptide group, as is also the finding of amino acids of the D series.

d. Polymyxins

Paper chromatography has been used extensively in the study of the polymyxins and, since it is perhaps only in this study that the very definition of the separate members of the group has been based on the findings of the technique, some expatiation is permissible. In 1947, the discovery of two antibiotics of somewhat similar properties was announced: " aerosporin " from *Bacillus aerosporus*, and polymyxin from *Bacillus polymyxa*. It was shown (Jones, 1948a) by paper chromatography that the former contained leucine, threonine and an unknown basic amino acid, the polypeptide nature of the antibiotic having been discovered by Catch (Catch & Friedmann, 1948). The unknown amino acid was proved to be αγ-diaminobutyric acid by comparison on paper chromatograms, and by direct comparison after isolation, with synthetic material (Catch & Jones, 1948). The application of Synge's method of determination on the paper chromatogram by treatment with D-amino-acid oxidase of the optical configuration of the leucine and threonine portions (Synge, 1949), showed the former to be of the D series and the latter of the L series (Jones, 1948b). Meanwhile the antibiotic itself was found to be capable of chromatography with a number of solvents, of which butanol–acetic acid mixtures were the most useful. Exchange of " aerosporin " and polymyxin was arranged, and the latter was found to contain the new αγ-diaminobutyric acid as well as leucine, threonine and serine. Of more importance was the finding that when chromatographed on paper, side by side or in admixture, the two antibiotics were clearly different. Both antibiotics had been found to suffer from a serious defect from the therapeutic point of view in that they were specifically toxic to the kidney (see Brownlee, Bushby & Short, 1949).

Workers at the Wellcome Research Laboratories examined many strains of *B. aerosporus* for their antibiotic production, and the paper chromatography of the products, both as the intact material and after hydrolysis, showed that they could be classified into groups. In the summer of 1948, at a conference convened by the New York Academy of Sciences, these facts were reported (Jones, 1949; Catch, Jones & Wilkinson, 1948, 1949) and compared with the results of the American workers (Bell *et al.* 1949). The latter had found that polymyxin contained D-leucine, L-threonine, L-αγ-diaminobutyric acid and D-serine. Although unconnected with chromatography, it may be mentioned that all the antibiotics contained a fatty acid, identified by Wilkinson (1949) as 6-methyloctanoic acid. The American workers also found that their polymyxin was composite, and could be fractionated by counter-current distribution and by partition chromatography at pH 2 on kieselguhr. A re-examination of the taxonomic basis of *B. aerosporus* showed that there was no justification for the differentiation from *B. polymyxa*, the more generally accepted name. Agreement was therefore reached by which the collective name for the antibiotics became polymyxin, " aerosporin " becoming polyxmyxin A; the polymyxin of Stansly, Shepherd & White (1947), polymyxin D; with the names polymyxins B, C and E assigned to further products of the Wellcome group (Brownlee & Jones, 1948). The chemical basis for the classification was given by Jones (1948c) and is reproduced as part of Table I. A chromatogram of the intact polymyxins on Whatman No. 4 paper, using butanol–15% acetic acid, is shown in fig. 2. It is seen that the polymyxins move as relatively compact spots and that those of identical qualitative amino-acid composition (A and E) are distinguishable in the intact state. Bell *et al.* (1949) determined the quantitative composition of polymyxin D by

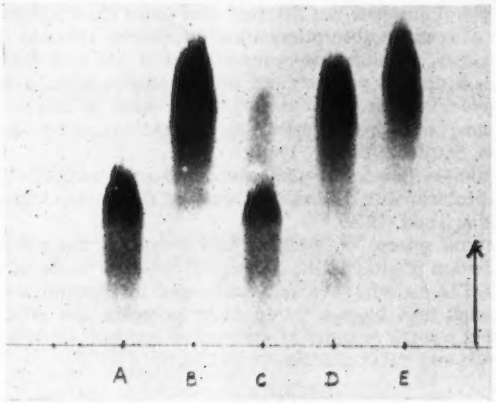
TABLE I. CONSTITUENTS OF THE POLYMYXIN GROUP OF ANTIBIOTICS

(Numerals indicate relative number of amino-acid residues present.)

Polymyxin	L-αγ-Diaminobutyric acid	L-Threonine	6-Methyloctanoic acid	D-Leucine	D-Phenylalanine	D-Serine	D-Valine	L-Isoleucine	Reference
A . .	+	+	+	+	–	–	–	–	Catch, Jones & Wilkinson (1949)
B . .	+	+	+	+	+	–	–	–	Jones & Wilkinson (unpublished)
C . .	+	+	+	–	+*	–	–	–	Jones & Wilkinson (unpublished)
D . .	5	1	1	1	–	1	–	–	Bell et *al.* (1949)
E . .	+	+	+	+	–	–	–	–	Jones & Wilkinson (unpublished)
Circulin .	5	1	1	1	–	–	–	–	Peterson & Reineke (1949)
Polypeptins A and B .	3	1	+†	2‡	1	–	1	1	Hausmann & Craig (1952)

* Assumed to be D-phenylalanine † Unidentified $C_6H_{10}O_2$ ‡ L-Leucine

FIG. 2. CHROMATOGRAM OF THE POLYMYXINS



Paper: Whatman No. 4, 16 hr.
Solvent: upper layer from *n*-butanol–15% (v/v) acetic acid (1:1 vol.)

conversion of the hydrolytic products to DNP-amino acids, which were separated by partition chromatography on kieselguhr and determined spectrophotometrically. The free amino groups were shown by Sanger's method to occur as four of the γ-amino groups of αγ-diaminobutyric acid. The absence of free carboxyl groups suggests that this antibiotic series is cyclic in nature, and a structure similar to that of gramicidin S may be written down (Bell *et al.* 1949).

As shown in Table I, two other antibiotics—circulin and polypeptin—may be considered as belonging to the polymyxin group. Circulin was isolated from cultures of *Bacillus circulans* and purified by charcoal chromatography. The amino-acid composition was determined (Peterson & Reineke, 1949) by cellulose powder chromatography. Polypeptin also contains αγ-diaminobutyric acid, but the fatty acid present is an octanoic acid (Hausmann & Craig, 1952).

Although the differentiation of the polymyxins was based on paper chromatography, some modification may be necessary in the future. It was considered at the time that one strain of organism produced one main antibiotic (Jones, 1949). It is now suspected that several members of the group are composite, apart from the certainty that polypeptin has been resolved into two, and circulin into five, constituents. The plurality of polymyxins B and E, in which renal toxicity is very much diminished (Swift & Bushby, 1953), cannot be regarded as having been satisfactorily settled.

Space does not permit the description of the chromatography of further antibiotic peptides; an excellent and comprehensive review containing details of a number of these is by Bricas & Fromageot (1953).

2. Vitamins

There is already such a large and growing literature on chromatography in this field that we can do no more than touch on a few interesting points. The classical method, depending upon physical adsorption, has been used extensively in the investigation of the fat-soluble vitamins A and D and of the carotenoids; details will readily be found in textbooks on chromatography. It was to the problems of vitamin A chemistry that the fruitful connexion between chromatography and ultra-violet spectroscopy first brought enlightenment in Great Britain; this has been continued in the work on the related pigments concerned in vision by Professor R. A. Morton and his collaborators. At the present time it might be considered more profitable to employ a reversed-phase method successful in the separation of the tocopherols (see, for example, Brown & Blaxter, 1951). A modern innovation, which should be mentioned in conjunction with alumina chromatography, is the use of paper impregnated with alumina for the separation of vitamin A alcohol and its esters (Datta, Overell & Stack-Dunne, 1949). Hardened filter-paper was dipped first into a solution of aluminium sulphate and then into aqueous ammonia, after which it was washed and dried. Non-polar solvents, for example, light petroleum, were suitable for development.

Ion-exchange methods have also been used extensively, particularly for the analytical separation of members of the B group, and also in their large-scale preparation (see bibliography by Osborne, 1953). These water-soluble substances usually contain basic groups which become attached to cationic resins and enable them to be concentrated, separated and quantitatively determined.

Paper chromatography, using aqueous solvents, and non-polar solvents in reversed-phase applications, has been found to be very useful in the analysis of most vitamins. One little-used application was the chromatopile. This device consists of a large number of filter-paper disks, clamped tightly together with their centres on a vertical axis and irrigated by siphoning solvent on to the uppermost. The material, applied to a number of disks near the top, becomes distributed in the usual elution bands, and individual disks may be removed for spot testing. Forrest & Todd (1950) used this method in their work on riboflavin and riboflavin 4′:5′-phosphate.

The vitamin group includes not a few which are specific growth factors for micro-organisms and much advantage has resulted, particularly in the detection of minute quantities on paper chromatograms. The method in this instance is to apply the dry chromatogram to the surface of a solid medium containing all the required nutrients except the particular essential component under investigation. The plate may have already been seeded with this organism, or the inoculum may be supplied after " printing ". Areas of growth after incubation denote the positions of the growth-promoting substances. Examples of this technique, named the bio-autographic method, could be multiplied; of these, Winsten & Eigen (1948) used *Saccharomyces carlsbergensis* as test organism for the separation of the B_6 components—pyridoxine, pyridoxal and pyridoxamine. Parallel with this is a description of the use of chemical agents for detection (Snyder & Wender, 1953). A variant of the method is to cut the chromatogram into small squares, which are then assayed microbiologically in test-tubes in liquid medium. Bowden & Peterson (1949) found this method useful in the analysis of hydrolysed liver extracts for biotin.

By far the most important and spectacular example of chromatography in the vitamin field was the discovery, separation and chemical analysis of the cobalamin (vitamin B_{12}) group. The discovery of vitamin B_{12} was announced independently in 1948 by Lester Smith and by Rickes, Brink, Koniuszy, Wood & Folkers. The former (Lester Smith, 1948) used charcoal and silica gel chromatographically in thin beds for the removal of the vitamin from concentrates, which were still extremely dilute mixtures of the active material. The progressive salting-out technique of Tiselius (1948) caused

sharpening of the red band due to the vitamin on the silica column. Partition chromatography on moist silica gel using butanol was one of the most important steps in concentrating the vitamin, leading to its isolation in crystalline form. Although the primary interest in vitamin B_{12} is for its activity against pernicious anaemia, it was found to be a growth factor for *Lactobacillus* spp., thus providing a convenient and rapid assay method independent of the human subject. Other chromatographic techniques have been extensively employed: the bio-autographic technique has been used for the examination of liver extracts and of cultures of *Streptomyces*, later found to be a more convenient source; multiple sheets and thick paper have been used for the preparative separation of the vitamin. The application of these various techniques, particularly to *Streptomyces griseus* fermentation liquors, has led to the recognition of at least three related members of the B_{12} group. A fourth member of the group, vitamin B_{12d}, was shown by Lester Smith (1952a) to be an artifact arising from lack of equilibrium of the moisture content of the silica and mobile solvent.

In the study of the degradation products of B_{12} and its congeners, paper chromatography has been of use in three distinct ways. Acid hydrolysis was found (Beaven, Holiday, Johnson, Ellis & Petrow, 1950) to produce three components, recognizable on paper chromatograms by quenching of the paper fluorescence excited by light of wave-length 254 mμ. After removal from the paper, the γ component was identified spectroscopically as 5:6-dimethylbenziminazole, of which the β component was found to be the 1-ribofuranoside, and the α component the 3′-phosphoryl derivative of the β component. Phosphate was detected after paper chromatography and determined absorptiometrically following removal from the paper. A ninhydrin-positive material was also detected in the hydrolytic products and, after confusion with 2-aminopropanol because of the identity of R_F values in four solvent systems, was finally shown to be D-1-amino-2-propanol (Ellis, Petrow & Snook, 1949).

Cellulose powder columns were also used successfully in this problem with analogous results by Buchanan, Johnson, Mills & Todd (1950).

Several groups of workers have examined the effect of irradiation of vitamin B_{12} by thermal neutrons in the atomic pile. The material is largely unchanged in appearance and, although held by one group to be complex, the products yielded a small quantity of radioactive B_{12}, with activity due to ^{60}Co and ^{32}P (Lester Smith, 1952b).

Epilogue

The introduction of new principles or the deployment of older techniques in novel conjunction is the occasion for the enthusiastic and intensive appraisal of their capabilities. During the period of the discovery and elucidation of the structures of the antibiotics and vitamins, the capabilities of chromatographic methods have been exploited fully and without delay. No chemist will dispute the indispensability of chromatography in these fields or the need for the widest dissemination of the knowledge of experimental devices and methods.

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CHROMATOGRAPHY OF STEROIDS AND STEROLS

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1 Special aspects of steroid chromatography
2 Adsorption methods (Table I)
3 Partition methods (Table II)
4 Miscellaneous methods
5 Methods of detection (Table III)
6 Methods of characterization and identification
7 Quantitative methods (Table IV)
8 " Gadgets and wrinkles "
9 Conclusion
References

The chromatography of steroids is of great medical interest because this group of compounds includes the steroid hormones and their metabolites, the cardiac glycosides, the saponins, cholesterol, the ergosterol group, and the bile acids. The biological importance of a group of compounds with such diverse pharmacological actions needs no emphasis here and it is interesting that a common carbon skeleton should be the basis of substances such as cholesterol, which has a structural function in animal cells, and the many different steroid hormones, drugs, and vitamins which by contrast fall into the class of biological catalysts. Methods of measuring such substances will always be of interest to clinicians and biochemists, and chromatography is already used extensively in existing methods for estimating steroids. Since they are a somewhat difficult group of substances for the uninitiated, generic or trivial names will be used where possible in this article and a survey of some typical steroids is given in fig. 1.

1. Special Aspects of Steroid Chromatography

At the outset it must be noted that steroids, like the larger peptides and proteins, often behave badly on chromatograms. This is due mainly to the following properties of steroids: (i) The molecular weights are rather large, ranging from about 290 in the oestrogens to 1,355 in the saponin, amolonin; (ii) all the steroids have a uniform stereochemistry with large relatively flat molecules; (iii) many steroids are unstable, being sensitive to light, oxidizing agents, strong acids and bases, heat, and strong adsorbents.

The first two properties tend to cause association between different steroids (and with other substances), low solubilities, and strong adsorption to various materials, including filter-paper. These all make for non-ideal behaviour in chromatographic systems (particularly partition systems (Martin, 1949a, 1949b)), while the third property is particularly important in adsorption systems where the concentration of ions and other substances at the surface of the adsorbent makes it difficult to predict or control the conditions to which steroids will be subjected in such systems. Thus benzene–water is seemingly a suitable solvent system for resolving adrenal steroids (Kendall, 1937) but is in fact unsuitable for partition chromatography because of the low water-solubility of many of these steroids and their adsorption by supporting materials like paper or silica gel (Bush, 1952). Again this group of steroids can be separated on alumina only in the form of their esters, since the free compounds suffer extensive re-arrangements and losses on this adsorbent (Reichstein & Shoppee, 1949), and Levy (1954) found that they are also partially destroyed on charcoal.

Several other factors cause practical difficulties with this group of compounds. Firstly, most steroids are neutral substances; this excludes one of the most useful variables in chromatography. Secondly, the majority have partition

FIG. 1. A SURVEY OF SOME TYPICAL STEROIDS

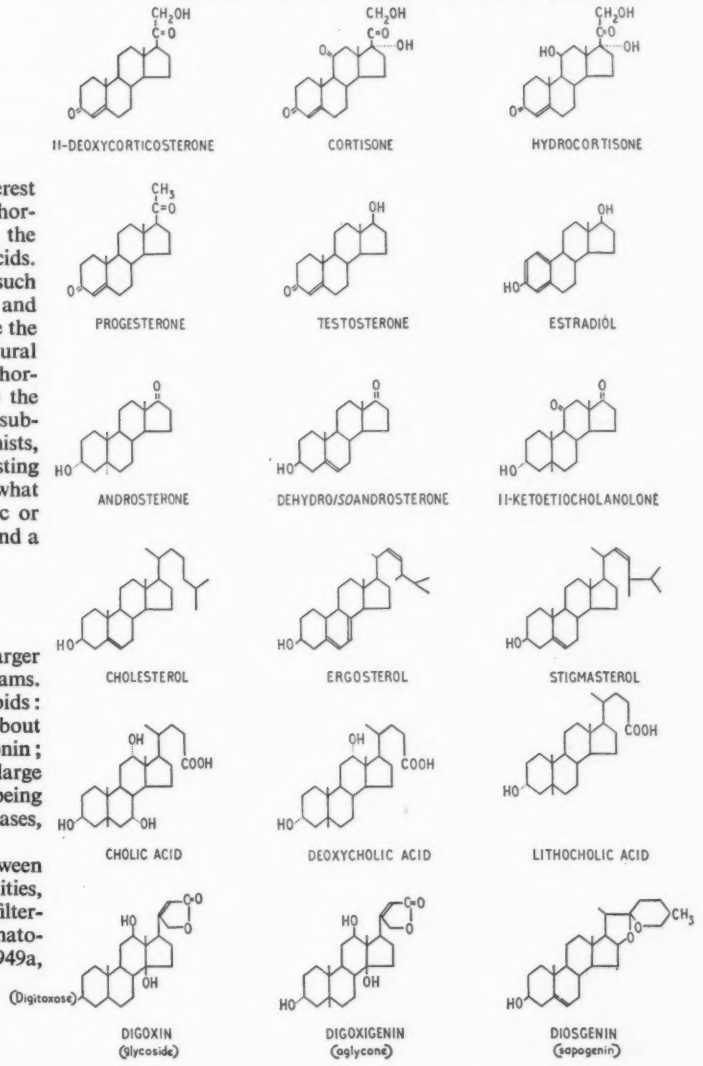

TABLE I. ADSORPTION METHODS

Method	Substance handled	Reference
Elution from alumina columns	Adrenal steroids	Reichstein & Shoppee (1949)
	17-Ketosteroids	Callow (1939)
	Oestrogens	Stimmel (1946)
	Oestrogens and pregnanediol	Stimmel *et al.* (1952)
	Urinary steroids	Dobriner & Lieberman (1948)
	Liver metabolites	Samuels (1947), Sweat & Samuels (1948), Wiswell & Samuels (1953)
	Kidney metabolites	West & Samuels (1951)
	Equine metabolites	Brooks & Klyne (1952, 1953)
	Steroid dinitrophenylhydrazones	Reich *et al.* (1952)
	Steroid dinitrobenzoates	Kellie & Wade (1953), Kellie *et al.* (1953)
Elution from alumina columns (microcolumn)	Urinary steroids	Dingemanse *et al.* (1946, 1952)
	Urinary steroids	Pond (1951), Edwards *et al.* (1953)
Gradient elution	Urinary steroids	Lakshmanan & Lieberman (1953)
Elution from alumina columns (microcolumn)	Cholesterol and esters	Kerr & Bauld (1953)
Elution from silica gel columns	Adrenal steroids	Pincus & Romanoff (1950)
Elution from silica gel columns (microcolumn)	Adrenal steroids	Sweat & Farrell (1953)
	Urinary steroids	Savard (1954)
Elution from florisil columns	Adrenal hormones in plasma	Nelson & Samuels (1952)
Elution from magnesium silicate columns	Adrenal hormones in plasma	Nelson & Samuels (1952)
	Urinary steroids	Lieberman *et al.* (1953)
Alumina paper (microcolumn)	Adrenal steroid esters, etc.	Bush (1950)
	Adrenal steroids, plant steroids, etc.	Shull *et al.* (1952)

coefficients very much in favour of the less polar phase of the commoner liquid–liquid systems. Thirdly, the mere number of known steroids and the large number of optical and geometrical isomers of each structural formula make it difficult to devise methods capable of dealing with all of them: in the simplest analysis of the problem it can be calculated that with a perfect two-dimensional method only 300 separate spots could be fitted on to one sheet of filter-paper of the usual size, and most column methods would require at least 1,800 fractions for the complete resolution of such a mixture. When one considers that human urine contains about 100 known steroids (Lieberman & Teich, 1953), each of which may occur free or as two known types of conjugate, the immensity of the problem is at once apparent. There is thus no convenient "map of the steroids" to work by, and instead it is usual to find workers in this field using a large variety of solvent systems and selective methods of detection to study only limited groups of steroids at each operation.

2. Adsorption Methods (Table I)

Alumina has been the mainstay of steroid chromatography for nearly twenty years, the method of its use being very similar to that in other fields. Apart from the classical work of Reichstein and his colleagues with adrenal extracts (Reichstein & Shoppee, 1943, 1949), it has also been used for the isolation of androsterone and etiocholanolone from urine (Callow, 1939) and in the epic work, on urinary steroids and steroid metabolism in man, of Dobriner and his group at the Sloan-Kettering Institute for Cancer Research, New York (Dobriner, Lieberman & Rhoads, 1948; Lieberman, Dobriner, Hill, Fieser & Rhoads, 1948; Dobriner & Lieberman, 1948). Micromethods for oestrogens and pregnanediol (Stimmel, 1946; Stimmel, Randolph & Conn, 1952) and for 17-ketosteroids (Dingemanse, Huis in't Veld & de Laat, 1946; Dingemanse, Huis in't Veld & Hartogh-Katz, 1952; Pond, 1951) also use alumina.

Two notable improvements in alumina chromatography of steroids have been introduced recently by Lakshmanan & Lieberman (1953). In the first place they have standardized their alumina by exposure to the atmosphere over a salt solution of known vapour pressure, and in the second place they have used gradient elution. A remarkable improvement in the sharpness and reproducibility of separations of urinary steroids was achieved in this way.

Silica gel (Pincus & Romanoff, 1950) and florisil (Nelson & Samuels, 1952) have been introduced for the chromatography of adrenal steroids in order to avoid the losses that occur when alumina is used (Reichstein & Shoppee, 1949). So far these adsorbents have been used only for relatively crude separations so that no comparison with alumina is possible. Their principal use has been in the rough separation of steroids into groups prior to paper chromatography (Savard, 1954) or in the application of a relatively specific colorimetric method which would not be usable with wholly unfractionated extracts (Nelson & Samuels, 1952). Silica gel is particularly useful for the rapid separation of steroids from fat and cholesterol in extracts of blood or plasma (Bush & Sandberg, 1953).

3. Partition Methods (Table II)

The advantages of this type of chromatography are discussed elsewhere and are particularly apparent when reproducible results with crude or complex extracts are desired with a minimum of time and trouble (Martin, 1949a, 1949b, 1950). The main problem with steroids has been to overcome the special difficulties mentioned earlier, principally their unfavourable partition coefficients in the commoner solvent systems and their tendency to be adsorbed by supporting materials, even filter-paper. An attempt to use more hydro-

TABLE II. PARTITION METHODS

Type	Supporting material	Solvent system: mobile phase–stationary phase, mutually saturated	Substances	Reference
I	Kieselguhr	Hexane–aqueous methanol	Progesterone, etc.	Butt *et al.* (1949)
		Toluene–aqueous ethanol	Adrenal steroids	Morris & Williams (1953a, 1953b)
		Heptane–acetic acid	Bile acids	Mosbach *et al.* (1953)
		Benzene–aqueous methanol	Pregnanetriol	Cox & Marrian (1953)
		Benzene–aqueous methanol	Aldosterone	Simpson & Tait (1953)
II	Filter-paper	Benzene–formamide	Adrenal steroids	Zaffaroni *et al.* (1950)
		Toluene–propylene glycol	Adrenal steroids	Zaffaroni *et al.* (1950)
		Toluene–propylene glycol	Cardiac aglycones	Schindler & Reichstein (1951)
		Benzene: chloroform–formamide	Cardiac aglycones	Schindler & Reichstein (1951)
		Ligroin–propylene glycol	17-Ketosteroids, progesterones, etc.	Savard (1952, 1953)
		*cyclo*Hexane–formamide	17-Ketosteroids, progesterones, etc.	Savard (1952, 1953)
		Toluene–propylene glycol	Cardiac aglycones	Heftman & Levant (1952a)
		Heptane–ethylene glycol phenyl ether	Sterols, etc.	Neher & Wettstein (1952)
		Chloroform–formamide	Adrenal steroids	Zaffaroni (1953)
		Ligroin–propylene glycol	Pregnane and *allo*pregnane derivatives	Rosenkrantz (1953)
III	Filter-paper	Ligroin: toluene–aqueous methanol	Oestrogen dyes	Heftman (1950)
		Ethyl acetate: chloroform–water	Cardiac glycosides, etc.	Jaminet (1950), Hassall & Martin (1951), Vastagh & Tuzson (1953)
		Ligroin: toluene–aqueous ethanol	Sapogenins	Heftman & Levant (1952b)
		Ligroin–aqueous methanol	Progesterone, etc.	Bush (1952)
		Benzene–aqueous methanol	Adrenal steroids	Bush (1952)
		Various similar systems	17-Ketones, etc.	Bush (1952, 1954)
		Various similar systems	Cardiac aglycones	Bush & Taylor (1952)
		Ligroin–methanol	Oestrogens	Mitchell (1953)
IV	Treated filter-paper	Aqueous ethanol–chloroform (esterified)	Sterols, etc.	Kritchevsky & Calvin (1950)
		Aqueous ethanol–chloroform (silicone-treated)	Progesterone, etc.	Kritchevsky & Tiselius (1951)
		Aqueous methanol–benzene (silicone-treated)	Progesterone	Edgar (1953a, 1953b)
	Untreated filter-paper	Water–butanol	Adrenal steroids	Hoffmann & Staudinger (1951)
Others	Filter-paper	Butanol–water	17-Ketosteroid conjugates	West *et al.* (1951)
		Aqueous K_2CO_3(?)	Oestrogens	Boscott (1951)
		Toluene–methanol: $CaCl_2.2H_2O$	?	Boscott (1952)
	Kieselguhr	Benzene–aqueous NaOH	Oestrogens	Swyer & Braunsberg (1951)
		Chloroform–aqueous NaOH	Oestrogens	Swyer & Braunsberg (1951)
	Silica gel	Hydrocarbons–nitromethane	17-Ketosteroids	Jones & Stitch (1953)
		Chloroform–ethanol	Adrenal steroids, etc.	Katzenellenbogen *et al.* (1952)

philic derivatives (Girard hydrazones) of ketosteroids gave poor separations because of the overwhelming influence of the hydrazide moiety (Zaffaroni, Burton & Keutman, 1949), and later work has been concentrated on four major lines of effort:

I. The use of kieselguhr columns with polar–non-polar solvent systems in which the stationary polar phase was aqueous methanol or ethanol (Butt, Morris & Morris, 1949; Morris & Williams, 1953a, 1953b) or acetic acid (Mosbach, Zomzely & Kendall, 1953).

II. The use of filter-paper impregnated with a non-volatile organic solvent as stationary phase (Zaffaroni, Burton & Keutman, 1950; Schindler & Reichstein, 1951; Neher & Wettstein, 1952; Savard, 1952, 1953; Axelrod, 1953a, 1953b).

III. The use of orthodox paper chromatography with volatile solvents for both phases (Heftman, 1950; Hassall & Martin, 1951; Bush, 1952; Mitchell, 1953).

IV. The use of reversed-phase systems using esterified paper (Kritchevsky & Calvin, 1950) or silicone-treated paper (Kritchevsky & Tiselius, 1951) as a hydrophobic supporting material.

Type I has been used extensively by Morris and his colleagues for the estimation of progesterone and adrenal hormones in blood, polarography being used for estimating the contents of the eluates from microcolumns. It is probably capable of separating all the steroids handled by types II and III, with the exception of the very hydrophobic sterols. It has already been used by Cox & Marrian (1953) for the estimation and isolation of pregnanetriol from human urine and by Simpson & Tait (1953) for the isolation of aldosterone. It is remarkable for the very close approximation to ideal behaviour that can be achieved (Morris & Williams, 1953b), but is at present rather laborious.

Types II and III have had the widest application and have been used for the widest range of steroids. Type II has been used successfully for adrenal steroids in extracts of glands (Zaffaroni & Burton, 1951), in human urine (Burton, Zaffaroni & Keutman, 1951), in human blood (Savard, Kolff & Corcoran, 1952), in adrenal venous blood (Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker & Pincus, 1951; Zaffaroni & Burton, 1953); for 17-ketosteroids and progesterones

TABLE III. METHODS OF DETECTION

Method	Substances detected	Approximate limit of sensitivity (μg.)*	Reference
Tollens reagent	α-Ketols	5	Zaffaroni *et al.* (1950)
Aqueous iodine	Cortisone and others	5	Zaffaroni & Burton (1951)
Iodine in petroleum	αβ-Unsaturated ketones, etc.	1–2	Brante (1949), Bush (1950)
Silicotungstic acid	Mainly 3-hydroxysteroids	5	Kritchevsky & Kirk (1952)
Phosphomolybdic acid	Mainly 3-hydroxysteroids	5	Kritchevsky & Kirk (1952)
Antimony trichloride	Mainly 3-hydroxysteroids	2–5	Kritchevsky & Kirk (1952)
Ultra-violet light	αβ-Unsaturated ketones	5	Haines & Drake (1951), Bush (1952)
Triphenyltetrazolium chloride	α-Ketols	3–5	Zaffaroni & Burton (1951)
"Blue tetrazolium"	α-Ketols and others	1–5	Chen & Tewell (1951), Simpson & Tait (1953)
Osmic acid vapour	αβ-Unsaturated ketones	2–5	Mancuro & Zygmuntowicz (1951)
NaOH fluorescence	Δ^4-3-Ketones	0.25–1.0	Bush (1952, 1954), Simpson & Tait (1953)
Phosphoric acid	A wide range of steroids	1–10	Neher & Wettstein (1952)
m-Dinitrobenzene and alkali	Cardiac aglycones, etc.	5	Schindler & Reichstein (1951)
m-Dinitrobenzene and alkali	17-Ketosteroids	2–5	Savard (1953, 1954)
m-Dinitrobenzoic acid and alkali	Cardiac aglycones	5	Bush & Taylor (1952)
2 : 4-Dinitrophenylhydrazine	Ketosteroids	3–5	Kochakian & Stidworthy (1952)
Diazotized sulphanilic acid	Oestrogens	2–5	Mitchell (1953)
Folin and Ciocalteu's reagent	Oestrogens	2	Mitchell (1953)
Trichloroacetic acid	Cardiac glycosides, etc.	5–10	Svendsen & Jensen (1950), Hassall & Martin (1951)
Infra-red spectra	$\Delta^{9(11)}$-Androstenolone, etc.	50	Dobriner & Lieberman (1948)

* This is stated in terms of the usual size of zone on a paper chromatogram, since figures in terms of quantity per unit area are mostly unavailable. Most zones are 1–3 cm.² in area in practice.

(Savard, 1952, 1953); for cardiac aglycones (Schindler & Reichstein, 1951; Heftman & Levant, 1952a); and for sterols and their esters (Neher & Wettstein, 1952). Type III has been used similarly for the analysis of adrenal extracts (Bush, 1952; Simpson & Tait, 1953; Schwarz, 1953a, 1953b), adrenal venous blood (Bush, 1952, 1953a, 1953b; Simpson, Tait & Bush, 1952; Bush & Ferguson, 1953), human urine extracts (de Courcy, Bush, Gray & Lunnon, 1953), human blood (Bush & Sandberg, 1953), adrenal slice metabolism in vitro (Haynes, Savard & Dorfman, 1952), progesterone in ovarian venous blood and corpora lutea (Edgar, 1953a, 1953b), cardiac aglycones (Bush & Taylor, 1952), steroid sapogenins (Heftman & Levant, 1952b), oestrogens (Mitchell, 1953), progesterones and 17-ketosteroids (Peterson, 1953; Bush, 1954), bile acids and esters (Haslewood, 1954), and bile salts (Haslewood & Sjövall, 1954).

Type IV has not yet found much application, despite its great technical interest, and the systems of Neher & Wettstein (1952) seem capable of handling the very hydrophobic sterols as efficiently as do the reversed-phase methods. Edgar (1953a) found it necessary to modify the method of Kritchevsky & Tiselius (1951) when working with extracts of blood or tissues.

4. Miscellaneous Methods

Partition chromatography on silica gel holding ethylene glycol (Haines, 1952) or ethanol (Katzenellenbogen, Kritchevsky & Dobriner, 1952) as stationary phase has been used for adrenal steroids, and Jones & Stitch (1953) have separated 17-ketosteroids on silica gel loaded with nitromethane in a promising method similar to that used by Ramsey & Patterson (1946) for separating the isomeric hexachloro*cyclo*hexanes.

The oestrogens, being phenolic, are one of the few groups of steroids in which ionic forces can be used to advantage. Swyer & Braunsberg (1951) have separated them on kieselguhr loaded with aqueous NaOH as stationary phase, and benzene and chloroform as mobile phase. Boscott (1951) has found that they and other urinary phenols can be separated on paper with the "single-phase" system (see Martin, 1950) of aqueous alkali. The convenience of this method is rather more obvious than its mechanism.

Several methods remain which—although not all of them have been used explicitly for steroids—are worth looking at since they may be of use in the steroid field and serve to put work on steroids in perspective with work on substances with similar solubility properties. The use of acetylated paper by Kostir & Slavik (1950) is interesting since it can be used equally well in the normal and in the reversed-phase manner. Another method of interest is that of Zimmerschied, Dinerstein, Weitkamp & Marschner (1950), who make use of the reversible reaction between hydrocarbons and urea for the chromatography of the former substances. Similar methods would be of great use in steroid work since the major limitation of present partition methods for steroids is that only very simple forces are made use of in the solvent systems described above. Thus, with very few exceptions (Table V), the order of mobility of different steroids is the same in all these systems (Savard, 1953; Zaffaroni, 1953; Bush, 1954) (completely reversed in the reversed-phase systems); this, to mention only one disadvantage, makes the use of *two-dimensional* chromatography on paper all but valueless. An extremely interesting approach to this problem has been described recently by Boscott (1952), who has used hydrotropic salts to increase the solubility of steroids in an aqueous stationary phase.

5. Methods of Detection (Table III)

An enormous number of these exist, so that it is perhaps better to discuss a few aspects of their use than to describe each one in detail. With few exceptions methods in general use have a limit to their sensitivity of about 0.3×0.6×

TABLE IV. QUANTITATIVE METHODS

Principle of method	Substances	Approximate range, etc. (μg.)	Reference
Elution from alumina, etc.	Oestrogens and pregnanediol (in urine)	Not stated	Stimmel (1946), Stimmel *et al.* (1952)
Zimmerman reaction on eluates from alumina columns	17-Ketosteroids (in urine)	20–250	Dingemanse *et al.* (1946, 1952)
Zimmerman reaction on eluates from alumina	17-Ketosteroids (in urine)	10–100	Pond (1951)
Zimmerman reaction on eluates from alumina after bismuthate oxidation	17-Ketosteroids, corticoids	5–100	Edwards *et al.* (1953)
Zimmerman reaction on eluates from partition columns	17-Ketosteroids	5–100	Jones & Stitch (1953)
Zimmerman reaction after elution from paper strips	17-Ketosteroids (in urine)	5–100	Rubin *et al.* (1953)
Polarography of Girard hydrazones, partition columns	Ketosteroids (in plasma)	0.25–20	Butt *et al.* (1949), Morris & Williams (1953a, 1953b)
Porter-Silber reaction after elution from florisil	17 : 21-Dihydroxycorticoids (in plasma)	0.25–20	Nelson & Samuels (1952)
Fluorimetry after elution from partition columns	Oestrogens	Not stated	Swyer & Braunsberg (1951)
Ultra-violet absorptiometry, elution from alumina	αβ-Unsaturated ketones (in homogenates, etc.)	Not stated	Samuels (1947), West *et al.* (1951), and others
Automatic ultra-violet absorptiometry of column effluent	αβ-Unsaturated ketones (in gland extracts, etc.)	10–1,000	Haines (1952)
Ultra-violet absorptiometry after elution from paper strips	Progesterone	1–50	Edgar (1953a, 1953b)
	Adrenal steroids	2–100	Zaffaroni & Burton (1953)
H_2SO_4 fluorescence on eluates from silica gel	Adrenal steroids	0.1–20	Sweat & Farrell (1953)
Formazans eluted after treatment of paper strips with triphenyltetrazolium chloride	Adrenal steroids	5–100	Hoffmann & Staudinger (1951)
Elution of molybdenum blue after treatment of paper strips with arsenomolybdate	Adrenal steroids	5–100	Schwarz (1953a)
Preparation of dinitrophenylhydrazones followed by alumina chromatography of products, ultra-violet absorptiometry	Progesterone, etc.	5–100	Reich *et al.* (1952)
Preparation of dinitrobenzoates followed by alumina chromatography, ultra-violet absorptiometry	Steroid alcohols	20–500	Kellie & Wade (1953), Kellie *et al.* (1953)
Alumina columns, Lieberman-Burchard reaction	Cholesterol and esters	Not stated	Kerr & Bauld (1953)

10^{-2} μmole/cm.² on a paper chromatogram or about 0.3×10^{-2} μmole/fraction in the eluates from a column. While the sensitivity of methods using reactions that are rapid in the cold for paper chromatograms probably allows little further improvement, the sensitivity of methods involving heat or more prolonged exposure to the reagents can probably be much improved in the future by modification of technique. Thus Kochakian & Stidworthy (1952) improved their results with dinitrophenylhydrazine by spraying on the reagent together with a blast of hot air. Similarly Bush (unpublished) found that the sensitivity of the fluorescence reaction of Δ^4-3-ketosteroids (Bush, 1952) was considerably increased by using aqueous (instead of ethanolic) NaOH to increase the duration of the reaction, and by adding a trace of triphenyltetrazolium chloride to quench the background fluorescence of the paper. Simpson & Tait (1953) have been able to detect 0.25 μg. (*ca* 0.5×10^{-3} μmole/cm.²) cortisone with this method, using photographic detection. The factors influencing colour reactions on paper are much more complex than those influencing reactions in solution (see, for example, Schwarz, 1953a) so that slight modifications of technique may produce great differences in sensitivity.

Many reagents used for detecting steroids are almost specific for particular types of steroids, but most of them leave much to be desired when their specificity with regard to non-steroid substances is considered. The traps laid for the unwary in this direction are well exemplified by Burstein's finding (personal communication) that one of the commoner antibiotics not only has the same mobility on paper chromatograms as hydrocortisone but also reduces triphenyltetrazolium chloride! Again, osmium tetroxide is a sensitive reagent for αβ-unsaturated ketones (Mancuro & Zygmuntowicz, 1951), but its common use in histology for detecting unsaturated fatty acids and lipids suggests that its specificity is low.

Most of the methods used with eluates from columns are used as a means of quantitative estimation, but the same limitations of specificity are found as exist in the methods used for paper chromatograms. They are discussed in section 7.

6. Methods of Characterization and Identification

The lack of specificity of many of the methods of detecting steroids and the enormous number of these substances have already been emphasized. These two factors have led workers in this field to place less emphasis upon chromatographic mobility as a means of identifying steroids than has been usual in other fields. Following the work of Dobriner, Lieberman, Rhoads, Jones, Williams & Barnes (1948), the infra-red spectrum has been of great use in the final identification of steroids separated by chromatography, but requires several milligrams of material and expensive apparatus. An alternative method was devised by Zaffaroni (1950) using the ultra-violet spectra in concentrated sulphuric acid, which needs only 50 μg. of material and which has been enormously useful. Bernstein & Lenhard (1953) have recently completed an extensive study of the spectra of 220 steroids in sulphuric acid, which will undoubtedly be of great use to others in the field and which describes the use of the method for quantitative estimation.

Another approach initiated by Zaffaroni & Burton (1951) is the use of small-scale chemical transformations to confirm

identifications. Adrenal steroids were eluted from chromatograms and re-run after acetylation, or after acetylation and oxidation with chromic acid. In each case reference samples of the expected reaction products were run in parallel with the material to be identified. Bush & Sandberg (1953) used the same principle when they used more extensive degradations to obtain the related 17-ketosteroids from adrenal steroids isolated from human blood.

7. Quantitative Methods (Table IV)

As with other methods, quantitative estimation using chromatographic separation involves a compromise between the efficiency of the separation that is carried out and the specificity of the method used for the final determination. The degree of compromise is further influenced by the source and quantity of material to be determined. Unfortunately, it is difficult for the average clinician or biochemist to discover from the original papers what sort of compromise has been struck in many of the methods available for steroid determinations. This makes it extremely difficult for him to assess the advantages or limitations of existing methods when he wishes to select one for his own use and often makes it impossible for him to judge whether or not an existing method can be modified or adapted to a new problem. It is a pity that these aspects of a quantitative method are not given more attention in papers describing such methods for steroids, since it is common practice in other fields, and because a considerable knowledge of steroid chemistry is needed to assess these methods in the absence of such information. In the paper of Nelson & Samuels (1952) describing their method for determining "17:21-dihydroxycorticoids", for example, little or no indication is given of the degree of separation achieved in their chromatographic step. In fact Eik-Nes & Bush (unpublished) found that the fraction taken for estimation by the Porter-Silber reaction is such a crude cut that it includes all the known active adrenal steroids and two of their principal urinary metabolites. It must thus include most of the quasi-steroid substances in human plasma described by Bush & Sandberg (1953); this probably explains the great improvement of this method effected by Bayliss & Steinbeck (1953) in their modification using a more appropriate blank solution in the final determination. This method in fact depends entirely upon the specificity of the Porter-Silber reaction (Porter & Silber, 1950), a fact that is of much importance when considering its use for other purposes or with other estimation methods and when interpreting results obtained with the method. The same criticisms apply even more stringently to the modification of this method by Weichselbaum, Margraf & Elman (1953), who use in addition the method of Mader & Buck (1952) for "α-ketols". By this means they claim to determine both hydrocortisone-like steroids and corticosterone-like steroids (i.e., lacking a 17-hydroxyl group). Not only was there no investigation of possible interference from non-steroid substances in plasma, but it is well known (Chen & Tewell, 1951) that "blue tetrazolium" (used by Mader & Buck, 1952) is appreciably reduced by steroids other than α-ketols.

These criticisms are intended as a warning to clinicians and non-specialists who are interested in making steroid determinations. Steroid methods can be compared with modern household cleansing agents: they are very powerful (according to the manufacturers) but they can be dangerous when mixed, and what works with "woollens" ruins "cottons".

The most elegant and reproducible quantitative method at present seems to be the kieselguhr column of Butt *et al.* (1949). While this method is rather lengthy in its original form using polarographic estimation (Morris & Williams, 1953a, 1953b), it is likely to be very versatile and has already been extended to spectrophotometric estimation (Simpson & Tait, 1953), and the chemical determination of pregnanetriol (Cox & Marrian, 1953).

Quantitative methods using paper chromatography cannot yet compete with columns but several promising methods have already been developed and used for 17-ketosteroids (Rubin, Rosenkrantz, Dorfman & Pincus, 1953) and adrenal steroids (Hoffmann & Staudinger, 1951; Schwarz, 1953a, 1953b; Romanoff, 1954). Hoffmann and Staudinger have devised the useful technique of spraying the paper with triphenyltetrazolium chloride and eluting the coloured reduction product with pyridine for colorimetric estimation, and Schwarz (1953a, 1953b) uses a similar method with arsenomolybdic acid.

The use of radioactive isotopes is not yet general but should be very useful in the sub-microgram range. Simpson & Tait (1953) have made the ^{14}C-labelled acetate of aldosterone

TABLE V. SOME EXCEPTIONAL DIFFERENCES IN CHROMATOGRAPHIC BEHAVIOUR

Substances	Behaviour on chromatograms	Reference
11-Hydroxysteroids and 17-hydroxysteroids	Relative mobilities reversed on changing from propylene glycol to methanol systems on paper. Separation of Compound B and *Substanz S* easier with former systems	Savard (1953, 1954), Bush (1952, 1954), Simpson & Tait (1953)
Aldosterone and cortisone	Relative mobilities reversed on changing from propylene glycol to methanol systems on paper and kieselguhr. Separated very much more easily in latter systems	Simpson & Tait (1953)
$\Delta^{9:11}$-Etiocholenolone and etiocholanolone	Separated in ligroin–aqueous methanol system on paper, but not in propylene glycol system. Partially separated on alumina	Bush, Engel & Spaulding (unpublished), Savard (1953, 1954), Rubin *et al.* (1953), Dobriner & Lieberman (1948)
Various 17-ketosteroids	Displacement effects in propylene glycol systems; some separations are sharpened by this, others prevented by association. Not found in methanol systems	Savard (1953, 1954), Bush (1954)

TABLE VI. SOME USEFUL TECHNIQUES IN STEROID CHROMOTOGRAPHY

Purpose	Principle of method	Reference
Detection of αβ-unsaturated ketones . .	Inhibition of fluorescence of a phosphor screen	Haines & Drake (1951)
Quantitative and even "spotting" of extracts on paper strips	Extract deposited over a liberal area and concentrated at origin by preliminary run with methanol, etc.	Bush (1952)
Extraction of steroid conjugates from urine .	Urine percolated through reversed-phase column of silicone-treated Celite holding butanol	Anderson & Warren (1951)
Removal of fats, etc., from plasma extracts before partition chromatography of steroids	Reversed-phase chromatogram with aqueous methanol–hexane on silicone-treated Celite column	Morris & Williams (1953a, 1953b)
Paper chromatography in small space, e.g., incubators	Paper strip run as spiral coiled on special former	Schwarz (1953b)
Removal of fats, etc., from plasma extracts before paper chromatography of steroids	Crude adsorption chromatogram on silica gel	Bush & Sandberg (1953)

and estimated it by direct counting on paper chromatograms. Bojesen, Keston & Carsiotis (1953) have extended their method for amino acids (Keston, Udenfried & Levy, 1947) to steroid alcohols by making the radioactive *p*-iodotoluenesulphonates.

In summary it can be said that quantitative chromatographic estimation of all the known steroids in amounts larger than 5 μg. has been achieved already or could be achieved by suitable combinations of known methods. Smaller quantities than this, however, present difficulties at present and existing methods for this range need further development before they are likely to be of general use.

8. "Gadgets and Wrinkles"

Gadgetry can easily assume the features of malignant growth, but some techniques of real use to anyone using chromatography for steroid work are given in Table VI. The most interesting are the use of reversed-phase chromatography for extracting steroid conjugates from urine (Anderson & Warren, 1951) and its use for removing fats from extracts of plasma containing steroids (Morris & Williams, 1953a, 1953b). The "chromatocoil" (Schwarz, 1953b) is of real use for reducing the space taken up by chromatographic apparatus.

9. Conclusion

An attempt has been made in this article to give clinicians and biochemists some idea of the range of methods available for steroid work, and to discuss the main divisions into which these methods can be arranged. In addition an attempt has been made to give an idea of the sort of factors which determine the suitability or otherwise of different methods for particular problems. Many details of economy, time, and materials have perforce been omitted but most of these will be found in original papers that have been quoted. The papers likely to provide the best introduction to this field are marked with an asterisk in the list of references.

It is instructive, in closing, to review some of the achievements in steroid biochemistry of recent years in order to see what a large part chromatographic methods have played in this field. Nearly all our knowledge of steroid metabolism (see, for example, Lieberman & Teich, 1953) and of adrenal steroids (Reichstein & Shoppee, 1943) depends very heavily on the use of chromatography, while our understanding of the biosynthesis of adrenal hormones (Hechter *et al.* 1951) and of adrenal secretion (Nelson, Reich & Samuels, 1950; Bush, 1953a, 1953b) was made possible largely by the development of new chromatographic methods. The power of the newer methods was perhaps most strikingly demonstrated by the elegance and simplicity of the experiments of Grundy, Simpson & Tait (1952), which culminated in the isolation of aldosterone (Simpson, Tait, Wettstein, Neher, von Euw & Reichstein, 1953; Mattox, Mason, Albert & Code, 1953). The rapid development of the industrial microbiological synthesis of cortisone also depended heavily on paper chromatography for the rapid screening of microbial reactions. All in all the contribution of chromatography to steroid biochemistry has not only been spectacular but in many ways crucial.

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* These references provide a useful introduction to most of the technical aspects of steroid chromatography for those unfamiliar with this field.

CHROMATOGRAPHY OF PROTEINS

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1 Adsorption chromatography
2 Ion-exchange chromatography
3 Partition chromatography
4 Paper chromatography
5 Conclusion
References

Specific adsorption has often been used in the purification of proteins, notably by Willstätter and his school, and nearly twenty years ago there were isolated examples of the application of chromatography to the fractionation of proteins, such as in the purification of catalase by Agner (1938). It is, however, only in the last five years, following the great development in the chromatography of small molecules, that extensive attempts have been made to apply similar principles to the separation of proteins. The subject has recently been reviewed by Zechmeister & Rohdewald (1951), Lederer & Lederer (1953) and Zittle (1953).

The principles employed have been adsorption (including salting-out adsorption), ion exchange, and partition. Considerable effort has also been directed to the chromatography of proteins on paper though the results have not been promising. The developments reported will be discussed under these four headings.

1. Adsorption Chromatography

Earlier work in this field (reviewed by Zechmeister & Rohdewald, 1951) was largely confined to what were essentially batch methods in that the required protein or the impurity was either completely adsorbed or not at all adsorbed. However, sufficient evidence was gained to establish that many enzymes could be adsorbed and eluted without loss of activity. A new principle has since been introduced by Tiselius (1948), which he named salting-out adsorption. It was observed that, while silica gel adsorbed little ovalbumin from aqueous solution, its adsorptive capacity for this protein was greatly increased in 2 M-ammonium sulphate, the adsorption being reversible. Phycoerythrin, insulin and pepsin behaved similarly and Shepard & Tiselius (1949) developed the method further. Serum albumin behaved as ovalbumin but the adsorption of serum globulin was little affected by changes in the salt concentration of its solution. Globulin and ovalbumin showed decreasing adsorption with the increasing alkalinity of the solution. When advantage was taken of these differences of behaviour, frontal analysis showed that, with a column of precipitated silica, kieselguhr and sodium phosphate (pH 7, $I = 0.1$), serum albumin and globulin could be separated; 20% of the serum albumin was irreversibly adsorbed. Swingle & Tiselius (1951) used tricalcium phosphate and found that the adsorption of phycoerythrin was greatly increased by as little as 0.1% NaCl in the solvent. With this adsorbent, phosphate in the buffer was preferentially adsorbed and hence displacement elution of the protein could also be effected. Serum albumin and amino-acid oxidase were chromatographed similarly, with a loss of activity in the latter case of 40% during chromatography.

Salting-out adsorption has been used in several other laboratories with some success and Polls & Schmukler (1953) were able, after preliminary purification by fractional precipitation, to use it to isolate and crystallize lactoperoxidase from skim-milk. Tricalcium phosphate and silica acid–Hyflo mixtures were the adsorbents. There was little adsorption from 0.01 M-K_2HPO_4 solution, it became marked at 0.1 M-K_2HPO_4, and elution occurred if the salt concentration was raised further to 0.5–1 M. The elution is possibly again due to displacement by the preferentially adsorbed phosphate. By taking restricted cuts and sacrificing some active material, considerable purification resulted from the chromatography and a crystalline product was obtained with an over-all yield of 15%.

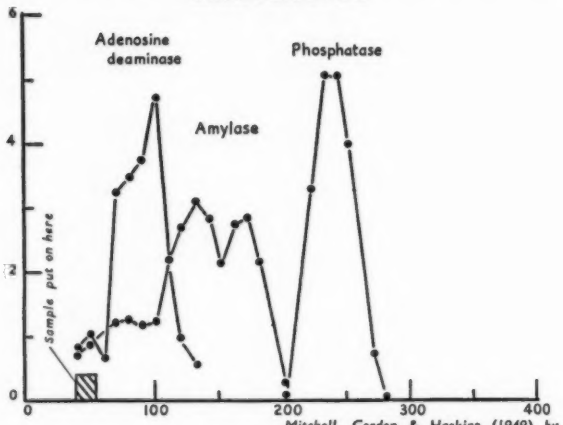
Rather remarkably, salt-promoted adsorption has been used in the purification of a virus and of enzymically active granules from liver cells (Riley, Hesselbach, Fiala, Woods & Burk, 1949; Riley, 1950). In both cases adsorption of the active material occurred if a solution in 0.9% NaCl was run through a kieselguhr column. Much impurity was not adsorbed and was therefore removed, the active fraction being subsequently eluted with water or weaker salt solutions. Twentyfold purification of the virus was achieved with this simple procedure.

Another method of chromatography inspired by salting-out adsorption has been developed by Mitchell, Gordon & Haskins (1949). Five hundred paper disks were clamped together in the form of a column named a chromatopile, 300 mg. of crude takadiastase were dissolved in water and the solution taken up in 20 disks of filter-paper which were inserted in the column 40 disks from the top. Sixty per cent saturated $(NH_4)_2SO_4$ solution was siphoned on top of the pile, while being continually diluted with distilled water, so that a solution of decreasing salt concentration flowed slowly through the filter-paper. After eight hours, when the liquid was reaching the lower end of the pile, the experiment was stopped and the enzymes extracted and assayed in five-disk sections. Figure 1 shows that considerable resolution of the different activities could be obtained. It is probable that both solubility and salt-promoted adsorption contribute to the separation of the enzymes.

2. Ion-Exchange Chromatography

Sober, Kegeles & Gutter (1949) made the first attempt to resolve proteins by ion-exchange chromatography. They used an aqueous solution of egg-white proteins at pH 6.8 and a column prepared from the sulphonic acid resin Dowex 50 (200-500 mesh) as the ammonium salt. Frontal analysis was used and the concentration of protein in the eluate was followed by observing the changes in refractive index, using the schlieren scanning technique. In this way a picture was obtained which was qualitatively similar to that given by electrophoresis of the same solution and showing the three components—ovalbumin, ovomucoid, and conalbumin. In a later paper (Sober, Kegeles & Gutter, 1952) it was noted, however, that solutions

FIG. 1. FRACTIONATION OF TAKADIASTASE ON A CHROMATOPILE



Mitchell, Gordon & Haskins (1949) by permission of Journal of Biological Chemistry

Abscissae: disk number
Ordinates: activity per 5 disks (% of original activity)

which had stood for several months showed only two components chromatographically, while being unchanged in the electrophoretic pattern. No explanation was offered of this anomalous behaviour. Mixtures of bovine plasma albumin and human carboxyhaemoglobin could be separated similarly, though resolution was dependent on the absolute and relative concentrations of the two proteins. The pH and ionic strength had relatively little effect in contrast to later work. As with salting-out adsorption there was evidence of a second component in the plasma albumin.

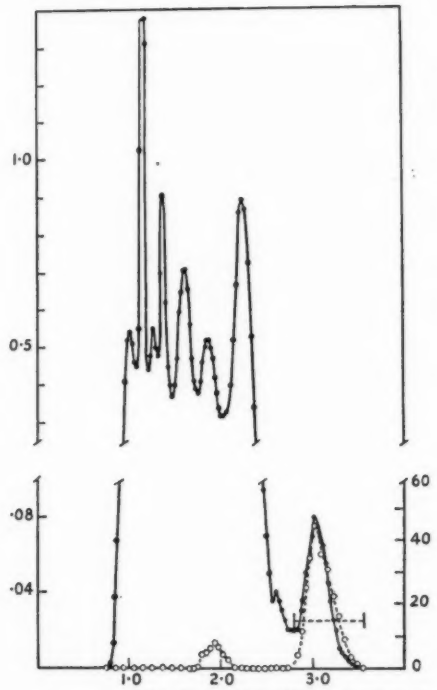
Paleus & Neilands (1950) introduced the use of the carboxylic resin Amberlite IRC-50 to protein chromatography, and this has been the resin used in all subsequent work. Starting with horse-heart cytochrome containing 0.34% iron, they were able to separate a yellow impurity and get three cytochrome fractions of which the largest, containing 0.46% iron, was considered to be as pure as any preparation so far obtained—or even more pure.

A systematic examination of a series of adsorbents and eluents was undertaken by Hirs, Moore, Stein and Tallan (Hirs, Moore & Stein, 1953; Tallan & Stein, 1953; Hirs, 1953). The adsorbents included Celite, starch, silica gel, tricalcium phosphate, benzoic acid, and a number of ion-exchange resins. By far the most successful was the Amberlite IRC-50. Before commencing chromatography, these workers found the distribution of the protein between the resin and the eluent and determined if the adsorption was completely reversible. When this was established, as it was for ribonuclease with phosphate buffer pH 6.47, lysozyme with buffer pH 7.18 and chymotrypsinogen with buffer pH 6.02, satisfactory chromatograms could be run. The enzymes appeared in the eluate at the volume calculated from the distribution coefficient, and recoveries of active material were high. Figure 2 shows a chromatogram with beef pancreatic ribonuclease in which a minor and a major component were found. Both components were found in the crystalline enzyme and in acid extracts of pancreas. This is in agreement with the results obtained by partition chromatography of the same enzyme.

Lysozyme (fig. 3), when freshly prepared as the carbonate, also showed a minor component, but on storage of the freeze-dried powder at room temperature this increased till it equalled the major component, and a third variant appeared, all being enzymically active. In solution at pH 9 and at 25° C. this transformation was more rapid, but the chemical or physical mechanism involved is not yet understood.

Chymotrypsinogen showed only one component, whether crystalline or in a crude extract of pancreas, although certain commercial preparations were heterogeneous, suggesting degradation during the preparation. Jollès & Fromageot (1953) used the same method to isolate lysozyme from rabbit spleen and found two active fractions, both with the same specific activity. An attempt has been made to fractionate haemoglobins similarly by using Amberlite IRC-50 (Boardman & Partridge, 1953) and, in fact, sheep foetal and bovine carboxyhaemoglobins, whose isoelectric points differ only by 0.5 pH units, could be separated. There was, however, con-

FIG. 2. SEPARATION OF RIBONUCLEASE FROM CRUDE EXTRACT OF BEEF PANCREAS, USING PHOSPHATE BUFFER pH 6.47 AND RESIN AMBERLITE IRC-50



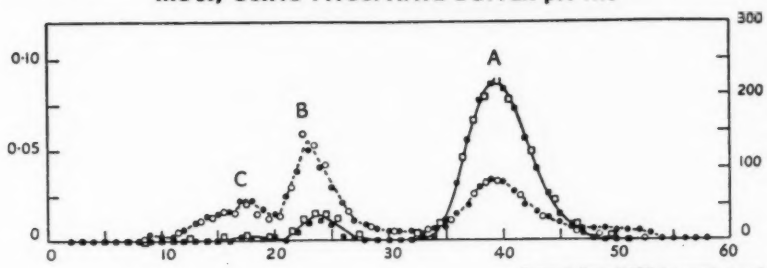
Hirs, Moore & Stein (1953) by permission of Journal of Biological Chemistry

Abscissae: volume of eluate (l.)
Ordinates:
Left-hand: ninhydrin colour value
Right-hand: ribonuclease concentration (Kunitz units/ml.)

—●— ninhydrin colour value

—○— enzyme activity

FIG. 3. FRACTIONATION OF LYSOZYME CARBONATE ON AMBERLITE IRC-50, USING PHOSPHATE BUFFER pH 7.18



Tallan & Stein (1953) by permission of Journal of Biological Chemistry

Abscissae: volume of eluate (ml.)
Ordinates:
Left-hand: ninhydrin colour value
Right-hand: activity units

A is the main component in freshly prepared lysozyme; B and C appear on storage

●——■ ninhydrin colour value
○——□ enzyme activity
□——■ freshly prepared lysozyme
○——● stored lysozyme

siderable loss by denaturation during chromatography. In this and all other work with the resin IRC-50 the pH of the eluent buffer is critical and exact control is essential.

It appears that, by the use of this resin, proteins which are relatively resistant to denaturation and have a basic isoelectric point can be chromatographed very successfully, and that with the development of new resins considerable extension of the method should be possible.

3. Partition Chromatography

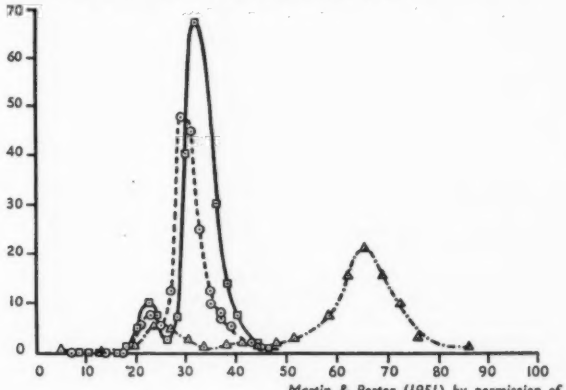
During the purification of catalase from *Micrococcus lysodiecticus*, it was observed (Herbert & Pinsent, 1948) that this enzyme could be partitioned between the two liquid phases produced by ammonium sulphate, water, and ethanol. With a suitable mixture the catalase went almost entirely into the ethanol-rich phase and considerable purification was obtained. This principle of partitioning proteins between the two liquid phases produced by water, a water-miscible organic solvent and a salt, was developed and applied to partition chromatography. By the use of ammonium sulphate, water and ethylene glycol monoethyl ether (cellosolve) to produce the phases and Hyflo as the support for the stationary phase, ribonuclease could be successfully chromatographed (Martin & Porter, 1951) and showed the same two components (fig. 4) as were observed with ion-exchange chromatography. Many other solvents and solutes were examined and a series of phase diagrams with a variety of characteristics prepared (Porter, 1954). It was found that insulin (Porter, 1953b) could be isolated in a single step from crude pancreatic extracts (fig. 5). Chymotrypsinogen and chymotrypsin (Porter, 1953a) could be separated from each other (fig. 6), and other enzymes such as trypsin and penicillinase could be chromatographed and separated from impurities.

The principal difficulty in the method lies in finding phase systems which give a suitable partition coefficient, say 5/1 to 20/1, while retaining sufficient solubility in the phase in which the protein is least soluble. As with all chromatographic methods there is risk of denaturing the protein by adsorption if this occurs. It seems, however, probable that partition chromatography could be extended to a considerable number of proteins and that it will have high resolving power.

4. Paper Chromatography

This type of chromatography has been the subject of more publications than all the other methods together. Some references are Franklin & Quastel (1949, 1950), Gross, Leblond, Franklin & Quastel (1950), Jones & Michael (1950), Swank, Franklin & Quastel (1950), Franklin, Quastel & Van Straten (1951), Hall & Wewalka (1951), Papastamatis & Wilkinson (1951), Sansone & Cusama (1951), Sansone, Cusama & Ravazzoni (1951), Boman (1952), Cabib (1952), Giri, Prasad, Gowri Devi & Sri Ram (1952), Kruh, Dreyfus & Shapira (1952), Robinson & Fehr (1952) and Tauber & Petit (1952). Little success, however, has been achieved and indeed it is doubtful if any of the methods described, which are essentially similar in that the solvents used are buffer solutions containing salt or organic solvent, could be claimed to be

FIG. 4. RESOLUTION OF CRYSTALLINE RIBONUCLEASE BY PARTITION CHROMATOGRAPHY, SHOWING INCREASING SEPARATION OF THE TWO COMPONENTS WITH DECREASING WATER CONTENT OF SOLVENT MIXTURE

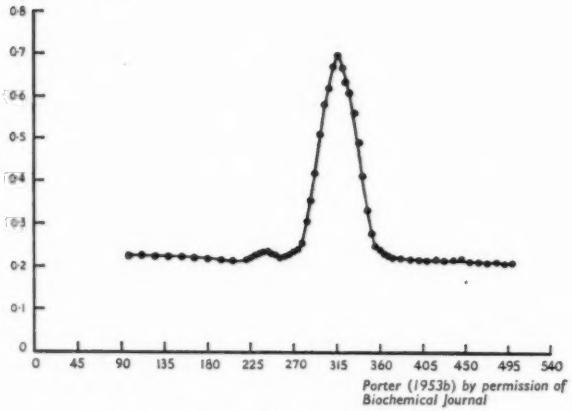


Martin & Porter (1951) by permission of Biochemical Journal

Abscissae: volume of eluate (ml.)
Ordinates: ribonuclease activity (Kunitz units)

Solvent mixtures:
⊙·····⊙ 15 g. $(NH_4)_2SO_4$; 30 g. cellosolve; 55 g. H_2O; 6 g. silica column
⊡——⊡ 20 g. $(NH_4)_2SO_4$; 24 g. cellosolve; 56 g. H_2O; 6 g. silica column
△·····△ 16.5 g. $(NH_4)_2SO_4$; 36.5 g. cellosolve; 47 g. H_2O; silica column

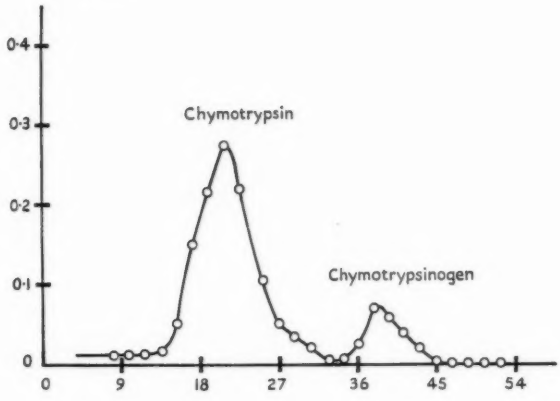
FIG. 5. ISOLATION OF INSULIN FROM CRUDE PANCREATIC EXTRACTS BY PARTITION CHROMATOGRAPHY



Porter (1953b) by permission of Biochemical Journal

Abscissae: volume of eluate (ml.)
Ordinates: absorption at 275 mμ.
Solvent mixture: 9 ml. 5 M-NaH_2PO_4(+ H_3PO_4 to pH 3.0); 6.67 ml. ethyl cellosolve; 3.33 ml. butyl cellosolve; 15 ml. H_2O
Impurities are stationary on the column; the trace peak shows hyperglycaemic activity

FIG. 6. SEPARATION OF CHYMOTRYPSINOGEN AND CHYMOTRYPSIN BY PARTITION CHROMATOGRAPHY



Abscissae: volume of eluate (ml.)
Ordinates: absorption at 280 mμ.
Solvent mixture: 32 ml. NaH_2PO_4(+ H_3PO_4 to pH 3.0); 17.33 ml. ethyl cellosolve; 8.67 ml. diethyl carbitol; 40 ml. H_2O

satisfactory. The objective clearly must be to obtain a chromatogram of a protein mixture such that reproducible discrete spots are apparent. Unless this is achieved, use of the method to determine if a given protein is present in a mixture, or if a purified protein still contains impurities, will be impossible. In most of the work quoted above, one-dimensional chromatograms show a trail extending varying distances from the origin and two-dimensional diagrams show areas of different shapes covered by protein. There would seem to be little prospect of separating similar proteins or of getting even an approximate estimate of the composition of a mixture. The impression so far is that a radical change of technique will be required before more than very limited application of such methods will be possible.

5. Conclusion

Of the methods described above, ion-exchange chromatography on Amberlite IRC-50 and partition chromatography have been used to isolate proteins from crude mixtures, and to resolve proteins which appeared to be pure by other criteria. Salting-out adsorption chromatography has also been applied successfully. The suggestion throughout has been that useful chromatography is confined to the proteins which are most resistant to denaturation, but this is a vague restriction. The value in biochemistry of methods of protein fractionation which have higher resolving power than the precipitational methods generally used is obvious, and wider application of chromatography to proteins is to be expected in the near future.

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SEPARATION OF AMINO ACIDS AND LOWER PEPTIDES BY DISPLACEMENT CHROMATOGRAPHY BY THE USE OF ION-EXCHANGE RESINS

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1 Displacement development with ion-exchange resins
2 Properties of the resin in relation to the displacement chromatogram
3 Separation of the amino acids of a protein hydrolysate
4 Separation of amino acids from biological extracts
5 Conclusion
References

The rapid extension of chromatographic method that is now taking place increasingly displays the diversity of solutes to be found in the watery fluids which comprise the bulk of living matter. Simple qualitative techniques such as paper chromatography are now available for the detection of almost every kind of hydrophilic solute of low molecular weight to be found in nature, and their increasing use reveals, almost daily, new substances of unknown chemical structure. Some of the most interesting of these compounds are present in very small amount, and their isolation and characterization by the methods of classical chemistry is often a matter of considerable difficulty. The characterization of a new compound often demands the isolation of at least a few grams, and if the substance is a minor component of the mixture the process of separation and purification involves the handling of large quantities of material. It is in these circumstances that the technique of displacement chromatography proves its value.

In the development of a displacement chromatogram about one-half or two-thirds of the column is first saturated with the mixture of solutes to be separated and a solution of a substance which is very strongly adsorbed on the column is then applied. This substance, the " displacement developer ", forms a " front " at the top of the column which displaces before it all the components of the original mixture in such a way that they form a progressive array of bands, the less strongly adsorbed components being displaced by components with higher adsorptive affinity. It will be clear that in such a system the maximum use is made of the adsorptive capacity of the column but, on the other hand, separation will not be entirely quantitative since the bands are contiguous and there must always be a certain degree of mixing at the boundaries. Thus, in general terms, the displacement chromatogram may be said to offer important advantages for the isolation of a substantial quantity of material; but where precise quantitative analysis on the milligram or microgram scale is envisaged, elution procedures are usually to be preferred.

Systematic studies of displacement chromatography have been carried out by Tiselius (1943) and Claesson (1946), who developed the technique at first as a micro-analytical procedure, using small columns packed with molecular adsorbents such as charcoal or alumina. When, however, it became necessary to increase the scale of the separations the obvious advantages of the newer synthetic ion-exchange resins as media for displacement chromatograms could not be overlooked, and in the work carried out in our laboratory between 1948 and 1953 the study was concerned solely with synthetic resins.

1. Displacement Development with Ion-Exchange Resins

Adsorption by ion-exchange resins proceeds by the exchange of an ion from the solution for an ion of the same charge which was previously held by the exchanger. This process is of the same nature as the " adsorption displacement " which takes place when an adsorbent such as charcoal or alumina is first saturated with one molecular species and then placed in a solution containing another. The modern monofunctional ion-exchange resins contain only one type of reacting group, and thus may be regarded as the ideal media for any chromatographic process which calls for complete adsorption displacement.

Let us consider as a typical case the behaviour of a resin prepared by sulphonating a cross-linked styrene polymer. Since the resin is a strong acid, it may be taken to be fully ionized in its hydrogen form. The reaction with a solution of a strong base may then be represented as follows:

$$R^- \ldots . H^+ + M^+ + OH^- \longrightarrow R^- \ldots . M^+ + H_2O.$$

This reaction is stoicheiometric and goes to completion: thus the amount of M^+ adsorbed will be independent of the concentration of MOH (except at very low values) and the " adsorption isotherm " will be a straight line parallel to the concentration axis. Adsorption of a very weak base will be rather different in character and will be lower at low concentrations of the base as a result of hydrolysis of the salt R^-M^+. In this case the relevant isotherm will be represented

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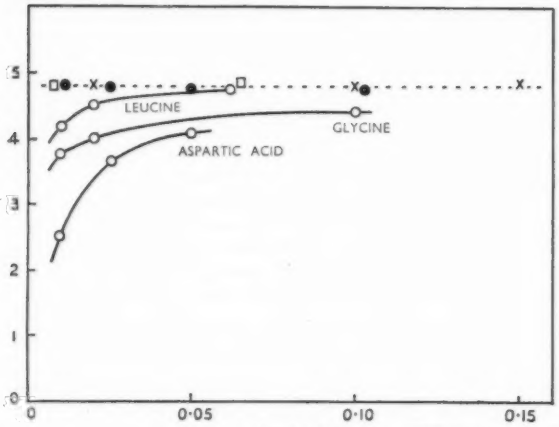
FIG. 1. RETENTION OF A NUMBER OF BASES AND AMINO ACIDS ON A SULPHONATED POLYSTYRENE RESIN



Partridge, Brimley & Pepper (1950) by permission of Biochemical Journal

Abscissae: concentration (mM/ml.)
Ordinates: retention (mM/g. dry Na^+ resin)

Dotted line: arginine -----□-----,
NH_3 -----x-----, NaOH -----●-----

by a very convex curve rising rapidly to a maximum value at high concentrations (fig. 1).

If we turn now to the adsorption of zwitter-ions such as the amino acids, these can be adsorbed only in their cationic form, but since the dissociation of an amino acid:

$$A^+ \rightleftharpoons A^{\pm} + H^+$$

is equivalent in a formal way to the dissociation of a base:

$$BH^+ \rightleftharpoons B + H^+$$

the amino acids may be regarded as weak bases and the adsorption behaviour of both types will be similar (Davies, 1949). Figure 1 shows the measured adsorption isotherms of a number of amino acids for a sulphonated polystyrene resin, compared with the isotherms for sodium hydroxide and ammonia (Partridge, Brimley & Pepper, 1950). In these experiments the amino acids were adsorbed from pure solution in which they exist substantially in their isoelectric condition. The highly convex form of the adsorption curves suggests that a system in which amino acids are adsorbed from isoelectric solution would be particularly suitable for separations based on displacement development. This has proved to be the case and it has now been shown that all the amino acids commonly present in the hydrolysis products of proteins can in fact be separated by displacement development through the use of ion-exchange resins.

With cation-exchangers, displacement of one amino acid by another depends mainly upon the depression of the ionization of weakly basic amino acids by those more strongly basic. Similarly, with anion-exchange resins the main mechanism is the depression of the dissociation of the weakly acid ampholytes by those with more strongly acidic properties. These relations are well brought out in Table I, which shows the experimentally determined order of displacement of a range of acids, bases and amino acids on both types of resin (Partridge & Brimley, 1952). In this table the amino acids marked with an asterisk fall out of the normal order based on the relevant p*K* values. It will be noticed that in each case the anomaly is always in the direction of retardation rather than acceleration, and it is thought that the effect is due to a larger contribution from short-range adsorption forces which may be associated particularly with ring structures.

2. Properties of the Resin in Relation to the Displacement Chromatogram

The cation-exchange resin that has so far proved most successful for the separation of amino acids is the Permutit product Zeo-Karb 215. The main features of this resin may be described by reference to the titration curve shown in fig. 2. The portion of the curve between pH 1 and pH 7 is the result of the titration of the sulphonic acid radicals of the resin, and that between pH 7 and pH 12 is the result of the titration of the phenolic hydroxyl groups. Thus between

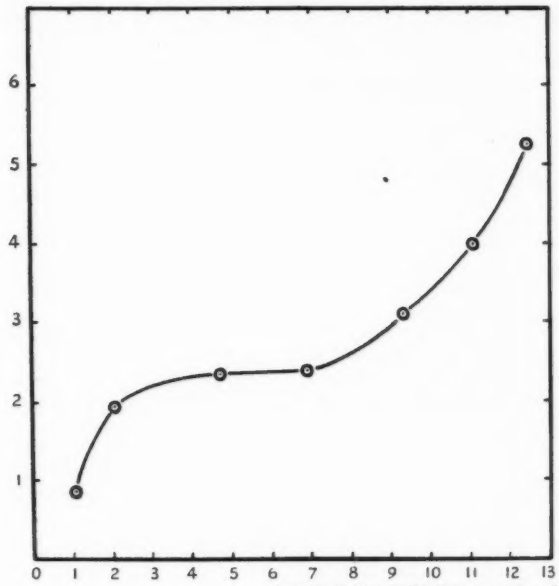
TABLE I. SEQUENCE OF DISPLACEMENT OF A RANGE OF SOLUTES FROM TWO RESINS AT ROOM TEMPERATURE

(The amino acids which form mixed bands in the routine procedures are bracketed together.)

Sulphonated cross-linked polystyrene (4.5% divinylbenzene)			Dowex 2		
Sequence of displacement	Value of relevant p*K*		Sequence of displacement	Value of relevant p*K*	
Aspartic acid	pK_1	1.88	Lysine	pK_3	10.53
Hydroxyproline	pK_1	1.92	Proline	pK_2	10.60*
Threonine	—	—	β-Alanine	pK_2	10.19
Serine	pK_1	2.21	β-Aminoisobutyric	—	—
Glutamic acid	pK_1	2.19	Alanine	pK_2	9.69
Proline	pK_1	1.99*	α-Aminobutyric	pK_2	9.60
Glycine	pK_1	2.34	Valine	pK_2	9.62
Alanine	pK_1	2.34	Leucine	pK_2	9.60
Valine	pK_1	2.32	Glycine	pK_2	9.60
Methionine	pK_1	2.28*	Carnosine	pK_3	9.51
Leucine	pK_1	2.36	Glutamine	pK_2	9.13
Cystine	pK_2	2.26*	Hydroxyproline	pK_2	9.73*
Creatine	pK_1	*ca* 3.0	Threonine	—	—
Phenylalanine	pK_1	1.83*	Serine	pK_2	9.15
β-Alanine	pK_1	3.60	Histidine	pK_3	9.17*
Trimethylamine oxide	p*K*	*ca* 4.5	Methionine	pK_2	9.21*
Creatinine	p*K*	*ca* 4.8	Asparagine	pK_2	8.80
Histidine	pK_2	6.0	Methylhistidine	pK_3	8.85
Methylhistidine	pK_2	6.48	Cystine	pK_3	7.85
Carnosine	pK_2	6.83	Phenylalanine	pK_2	9.13*
Anserine	pK_2	7.04	Tyrosine	pK_2	9.11*
Hydroxylysine	—	—	H_2S	p*K*	7.04
Lysine	pK_2	8.95	H_2CO_3	pK_1	6.46
Ammonia	p*K*	9.27	Acetic acid	p*K*	4.75
Arginine	pK_2	9.04	Glutamic acid	pK_2	4.25
Methylamine	p*K*	10.64	Aspartic acid	pK_2	3.65
—	—	—	Lactic acid	p*K*	3.85
—	—	—	Malic acid	pK_1	3.42
—	—	—	Citric acid	pK_1	3.08

*** Indicates amino acids which did not fall in the sequence based on p*K* values.**

FIG. 2. RETENTION OF NA^+ FROM A RANGE OF BUFFERS CONTAINING 0.053 N-NaOH ADJUSTED TO pH 12.1–1.1 BY THE ADDITION OF ACIDS



Partridge & Westall (1949) by permission of Biochemical Journal

Abscissae: pH of inflowing solution
Ordinates: retention (mM Na^+/g. Zeo-Karb (dry wt.))
Resin used was Zeo-Karb 215, H^+ form.

pH 1 and pH 7 the resin reacts as though it were monofunctional, but at higher pH values a range of weakly acidic groups is titrated.

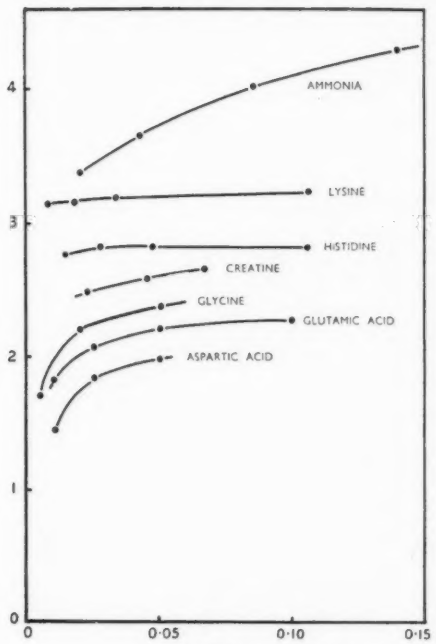
Figure 3 shows a number of adsorption isotherms obtained with various bases and amino acids. The adsorption of the various components is clearly determined to a large extent by the pH of the solution applied to the column, and the amount of each component adsorbed follows, more or less, what would be expected from the titration curve. All the natural amino acids are adsorbed from pure solution by Zeo-Karb 215, including aspartic and glutamic acids, the isotherms of which lie below that of glycine.

Obviously in displacement experiments the yield of pure component obtained depends upon the sharpness of the boundaries of the bands. The effect of various factors on the widths of the boundaries has therefore been studied in some detail (Partridge & Westall, 1949). The results of this work may be summarized in the statement that, with the resin Zeo-Karb 215, the boundary width is very roughly proportional to the rate of progression of the bands down the column except at very low flow-rates, when the boundary width tends to reach a constant value. The boundary width varies with a power of the particle diameter of the resin, but from the data obtained there appears to be no advantage, under practical conditions, in using the resin finer than 100–120 mesh/in.[1] In general, resin of this particle size has been used for fractionation experiments with small columns, but with longer columns, where the resistance of the resin bed to the flow of solution is an important consideration, 80–100 mesh or 60–80 mesh resin has been used. The rates of flow in such experiments must be adjusted to correspond with rates of progression of the boundaries of not more than 10–15 cm./hr.

With Zeo-Karb 215 it is found that, where strong bases are used, the overlap between bands becomes very wide. The reason for this effect of strong bases is clear from the titration curve given in fig. 2. At pH values above 7 the phenolic hydroxyl groups on the resin become ionized to an increasing extent. The ionization of these weakly acidic groups is accompanied by much swelling and is a slow reaction; thus in alkaline solution the resin behaves as though it were a mixture of two cation-exchangers, one reacting very rapidly and the other very slowly.

This feature of the resin is a disadvantage since it limits the range of pH over which practical operations are possible, but it is inherent in resins of the sulphonated phenol-formaldehyde type. The effect is sufficiently serious to reduce the sharpness of separations involving the important amino acids, arginine and lysine, and for this reason the use of sulphonated polystyrene resins, which contain no phenolic hydroxyl groups, has been investigated. Resins of this type are strictly monofunctional, but the rate of exchange depends critically on the

FIG. 3. RETENTION OF NH_3 AND A NUMBER OF AMINO ACIDS ON ZEO-KARB 215, H^+ FORM



Partridge & Westall (1949) by permission of Biochemical Journal

Abscissae: concentration (mM/ml.)
Ordinates: retention (mM/g. Zeo-Karb 215 (dry wt.))
Experiments were carried out with a column 8.5 mm. in diameter containing 1.44 g. resin (dry wt.).

[1] 1 in. = 2.54 cm.—ED.

degree of cross-linking of the polymer. Lysine, arginine and ammonia may be separated effectively by use of sulphonated polystyrenes (Partridge *et al.* 1950), but it proved necessary to use a lightly cross-linked form of the resin. Sulphonated polystyrenes containing only a small percentage of the cross-linking monomer, divinylbenzene, suffer large volume changes with changes in the ionic strength of the liquid phase, and for this reason the degree of cross-linking cannot be reduced below a certain rather critical value. For displacement chromatograms with amino acids and di- or tri-peptides a resin containing 4.5% (nominal) divinylbenzene has been found suitable. Such a resin (Permutit Co. Ltd., London) has a water regain (that is, a reversible absorption of water) at saturation of 1.8 g. water/g. of dry H^+ resin and a capacity of 5.3 mg. equiv. NaOH/g. dry H^+ resin.

The anion-exchange resin most frequently used for the separation of amino acids by displacement chromatography is the strongly basic bead-form resin, Dowex 2 (obtainable from R. W. Grieff & Co. Ltd., London). The resin is a cross-linked styrene polymer containing quaternary amine groups. In the chloride form the saturation regain is about 0.83 g. water/g. dry resin and its capacity for HCl about 3.7 mg. equiv./g. dry resin. Owing to the low water regain the exchange of amino-acid anions is relatively slow, and the resin should thus be used as fine-mesh beads (250–500 mesh/in.), and the rate of migration of the boundaries down the column should be restricted to 2–4 cm./hr. The resin absorbs CO_2 very strongly and in all experiments precautions must be taken to prevent the entry of atmospheric CO_2.

3. Separation of the Amino Acids of a Protein Hydrolysate

Since columns packed with synthetic resinous ion-exchangers may be regenerated and used again many times, it has become the practice to preserve packed filtration tubes as permanent pieces of equipment. In collecting together a set of ready-packed columns, there are obvious advantages in a certain degree of standardization as regards height and diameter of filtration tube. The use of coupled columns of diminishing diameter (Partridge, Westall & Bendall, 1947; Claesson, 1947) has now proved its value for most applications of displacement chromatography (Hagdahl, 1948) and the system has been adopted as a standard one in our laboratory. In addition to its great value in improving the sharpness of the boundaries, the use of standard column sections coupled together also leads to great flexibility. It has been shown empirically that the most advantageous column assembly for work with the majority of amino-acid separations consists of a set of three of the standard filtration tubes coupled in series (Partridge & Brimley, 1952). This scheme is flexible, since a set of three consecutive tubes can always be chosen to accommodate a given weight of mixture without incurring the waste of reagents and time that would be involved in using a column many times too large.

In order to effect the complete separation of a complicated mixture of amino acids and bases by a displacement method it is necessary first to carry out a " primary fractionation " in which the components are separated as a series of groups; the final separation of the components of the individual groups can then be carried out independently, for preference a resin being used with different properties from that used for the group separation. In most of the work on amino-acid separations carried out in our laboratory the procedure has been as follows. (i) The mixture of amino acids or amino-acid hydrochlorides is adsorbed on a column of sulphonated polystyrene resin and displaced with a solution of sodium hydroxide. This stage is included for the specific purpose of isolating histidine, lysine and arginine (fig. 4, bands VI–VIII); if these three amino acids or other strong bases are absent, or their isolation is not desired, this stage may be omitted. (ii) The fractions of the eluate from stage (i), containing the acidic and neutral amino acids, are combined, and the solution passed through a column of Zeo-Karb 215. The column is then displaced with dilute ammonia solution. This completes the " primary separation " and results in a series of mixed bands (fig. 4, bands I–V) which are very suitable in composition for the final separation by means of a strongly basic resin. (iii) Each of the mixed bands from stage (ii) is passed through a column of Dowex 2 and displaced with hydrochloric acid.

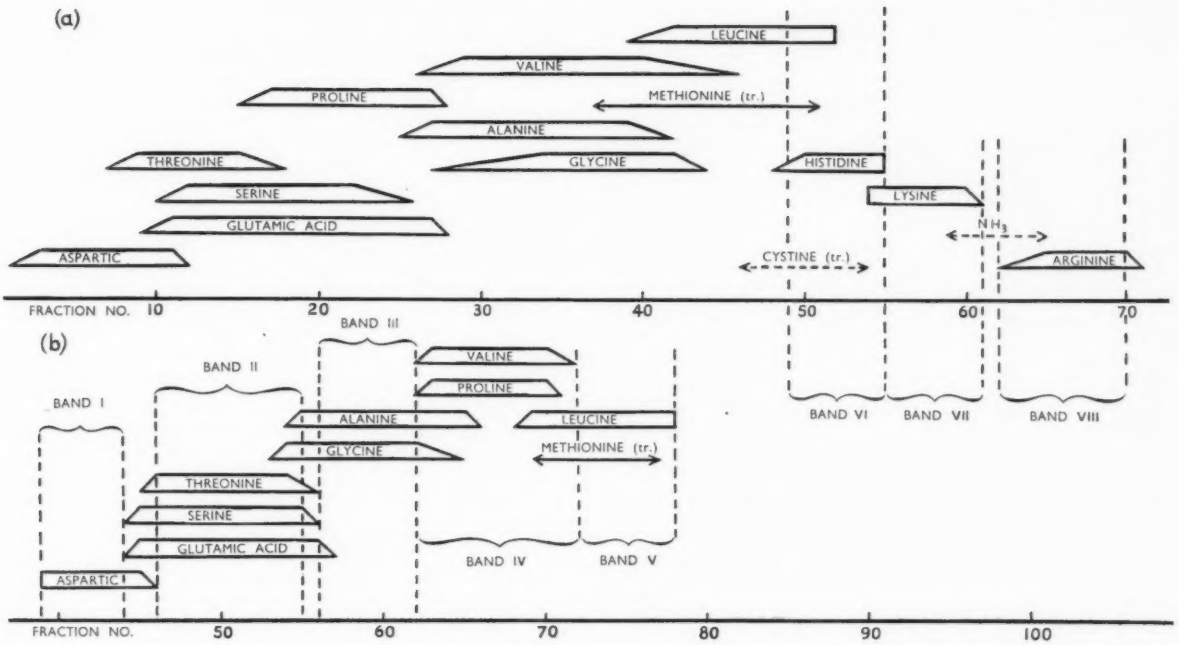
In all the experiments the separations are followed by collecting the effluents from the columns in a series of equal fractions by means of an automatic fraction collector. One drop of solution is then taken from each fraction and analysed qualitatively by the paper chromatographic method of Consden, Gordon & Martin (1944). Figure 4 is a graphical representation of the paper chromatograms obtained with the fractions of the column effluents during the " primary separation " of the hydrolysis products of 10 g. of yeast protein. The application of the displacement procedure results in the isolation of all the amino acids of protein hydrolysates except two pairs, which are not readily resolved.

TABLE II. YIELDS AND ANALYSES OF AMINO ACIDS ISOLATED FROM COMMERCIAL EGG ALBUMIN AND YEAST PROTEIN

Amino acid	Commercial egg albumin (280 g.)				Yeast protein (10 g.)	
	Yield (g.)	Percentage of protein (dry wt.)	Nitrogen (% found)	Nitrogen (% calc.)	Yield (g.)	Percentage of protein (dry wt.)
Aspartic acid	19.1	8.3	10.6	10.5	0.55	6.4
Glutamic acid	22.0	9.5	9.6	9.5	0.67	7.8
Serine	5.7	2.5	13.4	13.3	0.15	1.7
Threonine	4.9	2.1	11.6	11.8	—	—
Threonine + serine	—	—	—	—	0.43	5.0
Alanine	7.9	3.4	15.7	15.7	0.37	4.3
Glycine	3.3	1.4	18.4	18.7	0.20	2.3
Valine	10.6	4.6	11.9	12.0	0.41	4.8
Proline	2.3	1.0	12.2	12.2	0.26	3.0
Methionine	3.5	1.5	9.5	9.4	—	—
Leucine + isoleucine	18.8	8.1	10.5	10.7	0.23	2.7
Histidine	2.7	1.2	25.6	27.1	0.08	0.9
Lysine	6.8	2.9	12.9*	12.8	0.85	10.0
Arginine	7.8	3.4	31.7	32.2	0.56	6.5
Phenylalanine	5.0	2.2	6.9†	7.0	0.22	2.6
Tyrosine	4.4	1.9	7.8	7.7	0.15	1.7
Glucosamine	1.2	0.5	6.4†	6.5	—	—
Total	126.0	54.5	—	—	5.13	59.7

* Analysis as dihydrochloride
† Analysis as hydrochloride

FIG. 4. PRIMARY FRACTIONATION OF THE HYDROLYSIS PRODUCTS OF 10 g. OF YEAST PROTEIN



Partridge & Brimley (1952) by permission of Biochemical Journal

a: chromatogram prepared by displacing the mixture from a column of sulphonated polystyrene with 0.075 N-NaOH
b: fractions 1–49 from chromatogram (a) adsorbed on a column of Zeo-Karb 215 and displaced with 0.15 N-NH_3
(tr. = trace)

These are leucine-isoleucine and serine-threonine. The separation of the isomeric leucines has not been attempted, but, in view of the difficulty of obtaining pure L-serine and L-threonine by conventional methods, the separation of these two substances has been studied in some detail. After a number of trials, the most convenient procedure found consisted in displacing the mixture from a column of sulphonated polystyrene resin, using sodium hydroxide dissolved in 50% aqueous acetone as the displacement developer. Table II shows the yields and analyses of amino acids isolated from the hydrolysis products of two proteins.

4. Separation of Amino Acids from Biological Extracts

Biological extracts and secretory fluids usually contain substances of high molecular weight, such as proteins, polysaccharides or tannins, which must first be removed. The procedure adopted for removal of high molecular substances should not result in the addition of large amounts of inorganic salts to the extracts, and for small-scale work dialysis through cellophan, or the addition of ethanol to a concentration of about 80% (v/v), is suitable. Deproteinization may be followed by treatment with charcoal to remove phenolic substances, colouring matter and the aromatic amino acids. On a larger scale, the use of ethanol for deproteinization is inconvenient; the addition of a predetermined amount of basic lead acetate, following the procedure given by Westall (1950a), is preferable. Another procedure, which can be used either on a small or a large scale, involves the use of highly cross-linked ion-exchange resins as molecular sieves (Partridge, 1952). For instance, a highly cross-linked sulphonated polystyrene resin, in the form of large spherical beads, is almost unadsorptive towards proteins and other substances of high molecular weight, but rapidly takes up diffusible amino-acid cations, and these can afterwards be displaced from the resin by use of a strong base. The sulphonated polystyrene resins are particularly suitable in this respect, since other cation-exchange resins containing phenolic or carboxyl radicals in addition to $—SO_3H$ show a strong tendency to adsorb proteins on the surface of the particles, particularly from acid solution.

Once the components of high molecular weight have been removed from the mixture, the treatment applied may be substantially the same as that adopted for protein hydrolysate. The solution will usually contain inorganic salts, organic acids, sugars and other neutral substances in addition to the amino acids and bases; but on passing the mixture through a column of a strongly acidic cation-exchange resin in its hydrogen form, the inorganic cations remain at the top of the column while the organic and inorganic anions are not adsorbed and pass through as free acids together with the neutral substances. After it is washed with water, the column contains only cations

and ampholytes and, since the inorganic cations do not interfere with displacement when sodium hydroxide or ammonia solution is used as developer, the isolation of the organic bases and amino acids may proceed in the usual way.

5. Conclusion

The large range of commercially available ion-exchange resins makes them versatile reagents, and many of the resins have proved to be particularly suitable for chromatographic purposes. In the formulation of the commercial products, no deliberate attempt is made to introduce chemical configurations that are likely to confer specific adsorption effects, and the fact that the application of the displacement procedure has resulted in the separation of closely related homologues in the α-amino-acid series suggests that the method is capable of far wider application.

By judicious choice of resins and experimental conditions it has been possible to evolve a systematic procedure whereby almost all of the common amino acids can be isolated in a pure condition from complex mixtures. Needless to say, the same procedures will result in the enrichment of any hitherto unknown basic or amphoteric component in the mixture, and several unexpected or rare amino acids and bases have already been isolated from biological materials by these means. These include: γ-aminobutyric acid (Westall, 1950b; Hulme & Arthington, 1950), anserine (Shewan, Fletcher, Partridge & Brimley, 1952) and sarcosine (Dalgliesh, Johnson, Todd & Vining, 1950; Haworth, MacGillivray & Peacock, 1951). More recently Morrison (1953) has isolated L-pipecolinic acid from white clover and the isolation of γ-methyleneglutamine has been reported by Done & Fowden (1952).

The displacement procedure has found a particularly promising field of application in the hands of C. E. Dent and his colleagues at University College Hospital Medical School, London. During routine examination of normal and pathological urines by paper chromatography during the past five or six years (Dent, 1947, 1948), many ninhydrin spots were observed which could not be ascribed to any of the known naturally occurring amino acids or bases. An exact knowledge of the chemical constitution of these substances is of obvious importance in the study of normal and abnormal nitrogen metabolism, and a solution to the difficult problem of their isolation, prior to chemical characterization, has been found in the application of ion-exchange chromatography. Westall (1952) has published details of displacement procedures recommended for the separation of bases and amino acids present in urines, and the work has already resulted in the isolation of several of the new compounds. In preliminary communications the separation and identification of β-amino-*iso*butyric acid (Crumpler, Dent, Harris & Westall, 1951), methylhistidine (Searle & Westall, 1951) and ethanolamine (Dent, Fowler & Walshe, 1951) have already been reported, and recently Westall (1953) has made a further important contribution with the isolation of an S-hydroxyalkyl cysteine, " felinine ", from the urine of cats.

* * *

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AMINO-ACID METABOLISM

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1 The renal aminoacidurias
2 The " overflow " aminoacidurias
3 Phenylketonuria (phenylpyruvic oligophrenia)
4 Displacement chromatography using ion-exchange resins
5 Elution chromatography using ion-exchange resins
References

Earlier work on amino-acid metabolism was largely based on determinations of total amino nitrogen, using at first the nitrous acid (Van Slyke & Meyer, 1912), and later the more specific ninhydrin-carbon dioxide method (Hamilton & Van Slyke, 1943).

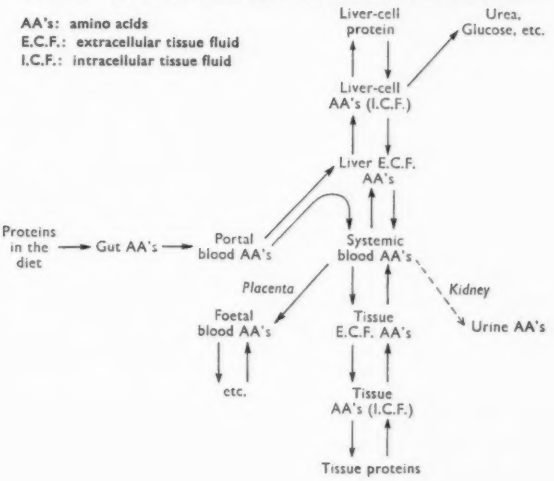
It was possible in this way to trace broadly the main features of amino-acid metabolism in the mammal, as summarized, for instance, in fig. 1 (Dent, 1954). This shows that the main source of our amino nitrogen is from dietary protein which is broken down in the gut to amino acids. These are absorbed as such into the portal blood, and enter into equilibrium with the various tissue proteins. They also undergo certain irreversible reactions such as are involved in the formation of urea, uric acid and urinary ammonia. There are many interesting details in these steps. For instance, the intracellular amino-nitrogen concentration is much higher than the extracellular (Christensen & Streicher, 1948). The fasting plasma level is kept fairly constant, *ca* 4.2 mg./100 ml. (Woodruff & Mann, 1945), and rises only 50–60% after a large protein meal. The rapid blood flow and the efficiency of cellular concentration, however, enable this low concentration to be adequate for tissue nutrition. There is also a small leak into the urine of about 1–2% of the dietary intake, and in the female the placenta can act as a "pump", for the foetal plasma amino-nitrogen level is always higher than the maternal (Christensen & Streicher, 1948; Crumpler, Dent & Lindan, 1950).

It is possible from a consideration of fig. 1 to make tentative suggestions as to the effects that certain diseases may have on amino-acid metabolism. Severe diarrhoea may, for example, lead to protein depletion, following defective protein digestion and impaired absorption of amino acids. A defective placenta may fail to "pump" amino acids into the foetus (Crumpler *et al.* 1950; Clemetson & Churchman, 1954) and thus cause its death or maldevelopment. Other abnormalities would be an excessive loss of amino acids into the urine or an inability of the cell to concentrate amino acids from the extracellular fluids. In all such cases, however, one realizes the inadequacy of investigations depending on a method which determines total amino nitrogen only. An excessive urinary loss of amino nitrogen (of, say, 10% of the dietary intake) would be of no consequence if it concerned all the amino acids in approximately the proportions present in the food, but would be very significant if it comprised mainly an essential amino acid such as methionine, since this would indicate that almost the entire supply was being lost and that, therefore, all the other amino acids could not be utilized for protein synthesis. It is in the further elucidation of such a problem that chromatography in all its forms has come to play a vital part, and can give us for the first time very full information about all the individual amino acids present in various fluids. In particular does this apply to paper chromatography (Consden, Gordon & Martin, 1944; Dent, 1948, 1951), which is the ideal micromethod for qualitative analysis of crude mixtures and, therefore, for the preliminary exploration of a new field of research. Displacement chromatography with ion-exchange resins, however, is also being used to identify unknown constituents of mixtures, and elution chromatography, also with ion-exchange resins (for references, see sections 4 and 5), has provided a fine tool for quantitative analysis. We shall now review a very few of the recent advances in chemical pathology made with the aid of such techniques.

The most fascinating feature disclosed by paper chromatographic analysis of the various body fluids has been the extreme diversity of the amino-acid pattern. This greatly lessens the value of amino-nitrogen determinations, and weakens the conclusions drawn from diagrams such as fig. 1. It also poses important theoretical problems. Why does sweat (Plate I, fig. A) contain a considerable amount of citrulline, an amino acid never present in more than traces elsewhere? Plasma (Plate I, fig. B) contains free amino acids in proportions approximately resembling those of the commoner animal proteins, except for the notably low amounts of glutamic and aspartic acids. Cerebrospinal fluid (Plate I, fig. C) is distinct from plasma because of its much lower concentrations of all the common amino acids except glutamine, which, therefore, appears in relatively large quantity.

In the study of disease, only plasma and urine have been extensively investigated by new chromatographic methods. Urine is the most accessible fluid, and disorders of urine amino-acid output have occupied much attention. These disorders occur as the result of two distinct mechanisms, the

FIG. 1. FATE OF AMINO ACIDS IN THE BODY



" renal " mechanism when the plasma levels are normal and the " overflow " mechanism when a rise in plasma level is the immediate cause of the increased excretion. These will now be dealt with in turn.

1. The Renal Aminoacidurias

A paper chromatogram of normal urine is shown in Plate I, fig. D. The pattern of amino acids is quite different from that in the plasma (Plate I, fig. B). It is of note that under basal conditions a particular individual shows a very constant urine pattern from day to day, but that different individuals, who appear to have the same plasma amino-acid levels, may show different urine patterns, for instance, that which we call " T-pattern ". This latter urine pattern is characterized by the excretion of large quantities of an unusual amino acid, β-amino*iso*butyric acid, which had not been described previously, and had to be isolated by ion-exchange chromatography and then identified by classical methods (Crumpler, Dent, Harris & Westall, 1951). Good evidence that the excretion of " T-pattern " is under genetic control has been provided by Harris (1953).

These individual differences are even more marked between different animal species (Datta & Harris, 1953): for instance, the domestic cat (Plate I, fig. E) excretes a large quantity of a new sulphur-containing amino acid, " felinine " (Westall, 1953), while a wild cat (Kenya genet) excretes a large quantity of cystine (Plate I, fig. F). So far as has yet been ascertained, these differences in animals also occur, as in man, in the presence of very similar plasma levels, and must, therefore, be due to innate differences of renal function. In man, there are congenital conditions in which more grossly abnormal patterns of amino-acid excretion occur. These may be symptomless or may be associated with more or less severe disease. For instance, in human cystinuria—the disease in which cystine stone formation occurs—four amino acids, cystine, lysine, arginine and ornithine, are excreted in quantities up to 50 times the normal (Plate II, fig. G). The subjects are quite healthy except for the tendency towards stone formation. In the Fanconi syndrome, child or adult type, the excretion is better called an aminoaciduria, since nearly all the common amino acids are excreted in excess (Dent, 1947a; Bickel & Smellie, 1952). In this case, other signs of kidney tubular damage are demonstrable both on histological grounds (Clay, Darmady & Hawkins, 1953), and by disorders of function, such as chronic loss of glucose and potassium, inability to pass an acid urine, proteinuria and polyuria. The patients suffer from severe symptoms of electrolyte disorders and usually from bone disease (Dent, 1952). At first sight these abnormal urinary amino-acid patterns appear as an irrelevancy of no significance, except as a valuable diagnostic sign, since quantitatively there is no serious loss of total amino nitrogen, nor of any particular essential amino acid. A further light is now being thrown on the possible immediate causes of a renal aminoaciduria. In Wilson's disease, another congenital disorder with aminoaciduria—this time affecting the nervous system—evidence is now coming forward which indicates that the kidney tubular damage is a rather late complication occurring only as the result of a chronic intoxication, possibly with copper. The primary factor may be a genetically determined deficiency of the plasma copper-binding protein, ceruloplasmin (Scheinberg & Gitlin, 1952). In another congenital disease, galactosaemia, there is evidence of a greatly decreased ability to metabolize galactose, leading eventually to cirrhosis of the liver and other changes. Here too an aminoaciduria has also been recently described (Holzel, Komrower & Wilson, 1952). Rather unexpectedly, this has also been shown to be of renal origin, and to be reversible, for it disappears when the patient is put on to a galactose-free diet, only to reappear when the galactose is again restored (Komrower, 1953). Clearly the significance of a particular renal aminoaciduria has to be considered carefully. If it is present from birth, it may indicate a disorder of tubule function closely related to the primary gene action, with possibly analogous abnormalities elsewhere in the body, but having been detected only in the kidney tubule because of its ready accessibility to investigation. On the other hand, another explanation is also possible, for a congenital disease can produce widespread disorders of cell metabolism, the manifestation of which may be sometimes delayed till adult life. A disturbance of kidney tubule function with renal aminoaciduria could be a late result of this, as in Wilson's disease. It is well known experimentally that various toxins, especially the heavy metals, can specifically damage the renal tubule. Examples of such acquired renal aminoacidurias in the human have already been reported in cases of poisoning by lead (Wilson, Thomson & Dent, 1953), and by Lysol (Spencer & Franglen, 1952). The hereditary aminoacidurias lend themselves readily to genetic studies, since it is possible to perform chromatographic analyses of urine from large numbers of relatives. This line of research is being actively pursued by Harris and his co-workers (Dent & Harris, 1951; Harris, 1953; Harris & Warren, 1953).

2. The " Overflow " Aminoacidurias

We will now deal with the aminoacidurias of the " overflow " type, due, that is, to increases in plasma amino-acid levels and not to disorders of renal function. It is hardly surprising in view of the classic work of Mann (1927) on hepatectomized dogs that this matter largely concerns diseases of the liver, the organ mainly responsible for maintenance of a constant plasma amino-nitrogen level. Mann and many others have stressed the large reserve of function that the liver possesses with regard to the deamination of amino acids, so it is not to be expected that gross disorders of amino-acid metabolism will occur unless the greater part of the liver has been destroyed. Early chromatographic work (Dent, 1947b) tended to confirm this, and showed that marked " overflow " aminoaciduria was found only in acute yellow atrophy of the liver, a condition in which fulminating massive necrosis of the liver occurred. Later, however, some modification of this view was reported and changes involving only a few amino acids (Plate II, fig. H) and related compounds were shown to occur in milder forms of liver disease, such as infectious hepatitis and cirrhosis (Dent & Walshe, 1951). It is of interest here to note the especial value of paper chromatography for such a preliminary survey. The substances found to be particularly sensitive to minor liver damage were cystine, taurine, β-amino*iso*butyric acid, methylhistidine, ethanolamine and methionine. These are all readily detectable on amino-acid chromatograms. Parallel studies have also been made, using more quantitative microbiological methods of investigation (Frankl & Dunn, 1947; Frankl, Martin & Dunn, 1947; Kinsell, Harper, Barton, Hutchin & Hess, 1948; Schreier, 1951; Gabuzda, Eckhardt & Davidson, 1952); but this method, however, is so far capable of analysing only methionine and cystine of the above compounds, so its value

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is restricted in these particular circumstances. The chromatographic finding of excessive outputs of ethanolamine was of particular interest in view of its key position in the synthesis of choline. Not only was ethanolamine often excreted with other amino acids in abnormal quantities in the urine in cirrhosis of the liver, but in one instance a very large excretion of the order of 0.5–1.0 g./day was found in the urine in the absence of other abnormal changes (Dent & Walshe, 1953). This patient, who had a huge hepatoma, had normal liver function as determined clinically and by the usual biochemical tests. The liver had no detectable cirrhosis in its remaining parts. Preliminary studies suggested that the ethanolamine was produced by the tumour cells. There did not appear to be any evidence of defective choline synthesis.

The earlier findings (Dent & Walshe, 1951) have recently been confirmed and extended by a study of over 100 cases of various liver diseases (Walshe, 1953a). Very large numbers of chromatograms of plasma, urine and cerebrospinal fluid were prepared from these cases. The general thesis that the aminoaciduria of liver disease was due to a raised blood level was fully confirmed (Plate II, fig. J), likewise the non-specificity of the amino-acid analysis as a means of detecting minor degrees of liver dysfunction. Of especial interest was the fact that, in the case of amino acids or related compounds (e.g., cystine, taurine, β-amino*iso*butyric acid and ethanolamine) with relatively high renal clearances, the urine changes were often more striking than those in plasma. However, those with lower clearances (e.g., methionine, phenylalanine and tyrosine) showed greater changes in the plasma or cerebrospinal fluid (Plate II, fig. K), since the urine excretion might increase but little following a relatively large rise in plasma concentration. Of some clinical value was the fact that patients with secondary carcinoma of the liver, obstructive jaundice and liver infiltrations had no abnormality of amino-acid metabolism. The extent of abnormality found in parenchymatous disease of the liver did vary roughly with the extent of the disease, both in the same patient and from patient to patient. Of the cases of acute yellow atrophy studied, one patient recovered with a coincident return to normal of his urine amino-acid excretion (Plate II, figs. L, M and N). This corrects the earlier view (Dent, 1947b) that such a condition was inevitably fatal. The claim (Walshe, 1951) that methionine sulphoxide, a glutamic acid antimetabolite (Borek, Miller, Sheiness & Waelsch, 1946), was present in the plasma of a patient dying in hepatic coma, led to a suggestion that this might be interfering with brain metabolism, glutamic acid being the only amino acid readily metabolized by the brain (Waelsch, 1949; Weil-Malherbe, 1950). Preliminary attempts to treat the coma of cirrhosis with glutamic acid have given encouraging results (Walshe, 1953b).

Amino-acid disorders in liver disease have also been explored by Blass, Cachin & Durlach (1950), using paper chromatography. These authors analysed only the plasma and stressed the raised levels of tyrosine usually found. In experimental animals, Flock, Mann & Bollman (1951) investigated plasma amino acids in the dog before and after hepatectomy. Ferrari & Harkness (1951) studied the free amino acids in the liver cells of the rat before and after partial hepatectomy. They found a large rise in ethanolamine phosphate during the phase of rapid liver-cell regeneration, a finding reminiscent of that reported by Flynn & Walshe (1951), who studied the urine of a man who had a resection of the left lobe of the liver. An interesting " artifact " was discovered by Walshe (1953a), in his study of urine from cases of liver disease. An unknown sulphur-containing amino acid was found frequently in the urine. It behaved like a higher homologue of cystine. It was shown to be penicillamine, (ββ-dimethylcysteine), which was derived from the penicillin given therapeutically to the patients.

3. Phenylketonuria (Phenylpyruvic Oligophrenia)

In this condition there is an " overflow " aminoaciduria of congenital origin involving only one amino acid, phenylalanine. Earlier speculations, to the effect that it was the result of the absence of an enzyme in the liver that was responsible for the oxidation of phenylalanine to tyrosine, have recently been confirmed directly on post-mortem material (Jervis, 1953). Recent work has been directed to determine what remaining metabolism, if any, of phenylalanine could be effected by the phenylketonuric patient. For this, paper chromatography is being mainly used. Woolf (1951) has found in the urine a phenylacetic acid derivative of glutamine. Boscott & Bickel (1954) are now studying extensively the aromatic acids in the urine and have claimed the presence of larger amounts than normal of degradation products of *o*- and *p*-tyrosine.

4. Displacement Chromatography using Ion-Exchange Resins

This method was first utilized for amino-acid isolations by Partridge & Westall (1949), and has been improved and extended in the later publications from their laboratory (summarized by Partridge & Brimley, 1952). Although overlapping of amino-acid bands occurs in the chromatographic column, which somewhat lowers the yields of pure amino acids from each fractionation as against an elution method, the displacement method is supreme from the point of view of its large scale and comparative simplicity of operation. A column, for instance, of the large size (100 cm. × 7 cm.) required for isolating 30–100 mg. of a pure amino acid by the elution method (see section 5), may be used to isolate a thousand times more when its capacity is being fully exploited by the displacement method. It is to be hoped that industry will overcome its apparent reluctance to use such a method for preparing the natural forms of the amino acids at reasonable cost. Westall (1952a) has applied this method extensively to human and animal urine, and has isolated in pure state three new amino acids, β-amino*iso*butyric acid (Crumpler *et al.* 1951), felinine (Westall, 1953), and 3-methylhistidine (see section 5), and in addition, the pyrrole derivative, porphobilinogen (Westall, 1952b). Westall (private communication) is currently analysing a sample of 100 l. of urine from a normal man. Paper chromatograms run on concentrated fractions indicate the presence in this urine of at least 32 unknown ampholyte ninhydrin reactors, of which ten are resistant to hydrolysis with 5.5 N-HCl for 16 hours at 100° C. In addition, there are 29 substances identified chromatographically as being already known amino acids. It is clear that our views on amino-acid metabolism will have to be profoundly modified and extended as more and more of these unknown substances are identified. The acid-labile fractions are of particular interest, since some at least of these are small peptides, and on theoretical grounds it is more likely that they are hormones (or their immediate degradation products) or other physiologically active substances, rather than presumably inactive (and less interesting) fragments of body proteins

lost from the cells during the processes of synthesis and degradation.

Urine amino-acid analyses by essentially similar methods are also being carried out by Boulanger, Biserte & Courtot (1952).

5. Elution Chromatography using Ion-Exchange Resins

As developed by the Herculean labours of Moore & Stein (1951), this has become by far the best method for the complete and accurate quantitative micro-analysis of amino acids in biological fluids. It is necessarily tedious and its application to medical problems has to be correspondingly restricted. However, analyses confirming and greatly amplifying the paper chromatographic findings are beginning to be reported. Stein (1951) has confirmed the large excretions of lysine, arginine and ornithine, as well as of cystine, in cystinuria in man. His finding of ornithine was particularly important, since the paper chromatograms were rather ambiguous on this point (Dent & Rose, 1951). The excretion of amino acids in normal urine has been determined (Stein, 1953). It is interesting that the elution curves obtained in all these cases also show the presence of large numbers of unknown amino acids and peptides, in agreement with Westall's findings (see section 4).

Elution chromatography has recently been used for preparative work. Hirs, Moore & Stein (1952) have been able, using large columns, to prepare very pure samples of amino acids in 30–100 mg. amounts. This has already been applied to isolate and identify one of the "unknowns" in normal human urine. This was shown to be 3-methylhistidine (Tallan, Stein & Moore, 1954), an isomer of the better-known constituent of anserine, 1-methylhistidine, which latter also occurs in the free state in normal urine (Searle & Westall, 1951). After an exchange of samples their new 3-methylhistidine was shown to be identical with a pure compound isolated, but at that time not yet identified, by Searle and Westall from cat urine.

In conclusion one can only remain amazed at the progress made in the last ten years in the techniques of amino-acid analysis. Almost any biological fluid can now be analysed qualitatively by a method of great specificity and simplicity, only microgram quantities of amino acid being necessary for detection. Using slightly larger quantities, an accurate quantitative method is now available which determines each constituent independently. If an unknown substance is present to the extent of 20–50 mg. as a minor constituent in a mixture of great complexity containing many other compounds of similar structure and properties, it can now be isolated almost certainly in pure state and in good yield. One can reasonably expect great advances to be made soon in our knowledge of normal and abnormal amino-acid metabolism.

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AMINO-ACID METABOLISM

(FIGS. A—N)

C. E. Dent & J. M. Walshe

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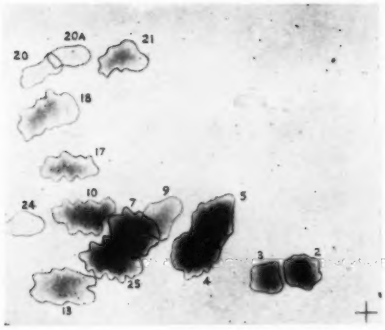

FIG. A. Normal human sweat, 125 μl. Note the high amino-acid concentration, particularly the very large citrulline (25) spot

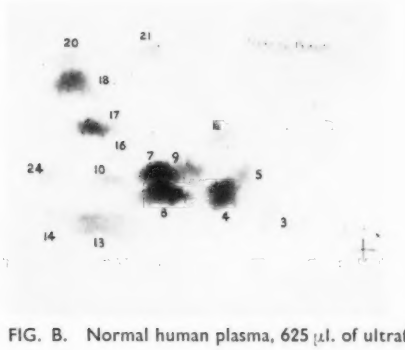

FIG. B. Normal human plasma, 625 μl. of ultrafiltrate

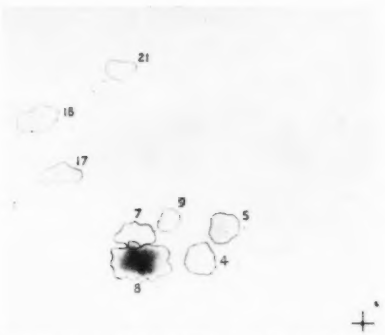

FIG. C. Cerebrospinal fluid, 625 μl. of ultrafiltrate. When compared with plasma, glutamine is present in much higher concentration relative to the other amino acids

FIG. D. Normal human urine. This is the common pattern, with glycine as the dominant amino acid

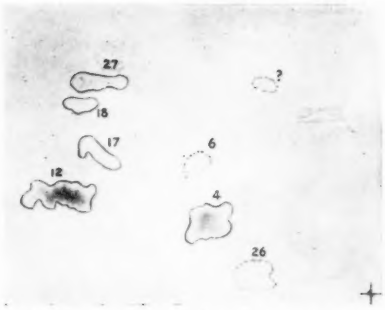

FIG. E. Urine of the domestic cat, showing the new sulphur-containing amino acid, "felinine" (27), and also the large excretion of methylhistidine (12)

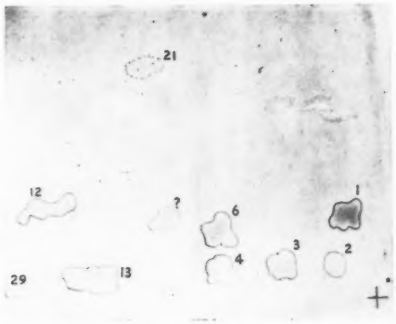

FIG. F. Urine of a wild cat, the Kenya spotted genet. There is a large excretion of cystine (1) with only a little lysine (13)

In each case the solution under test (urine or ultrafiltrate of plasma or cerebrospinal fluid) has been placed on the cross shown in the lower right-hand corner of figure; phenol was run from right to left as the first solvent, followed by collidine–lutidine upwards as the second solvent; the amino-acid colour reactions were then developed with 0.1% ninhydrin in butanol.

1: cysteic acid (from cystine); 2: aspartic acid; 3: glutamic acid; 4: glycine; 5: serine; 6: taurine; 7: alanine; 8: glutamine; 9: threonine; 10: histidine; 11: β-amino-*iso*butyric acid; 12: methylhistidine; 13: lysine; 14: arginine; 15: γ-aminobutyric acid; 16: α-amino-*n*-butyric acid; 17: valine; 18: leucine or isoleucine; 19: ethanolamine; 20: phenylalanine; 20A: tryptophan; 21: tyrosine; 22: methionine; 23: methionine-sulphone; 24: proline; 25: citrulline; 26: phosphoethanolamine; 27: "cat spot" (felinine); 28: artifact due to chloride; 29: artifact due to protein

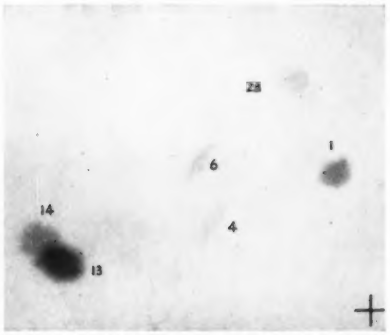

FIG. G. Urine from a patient with cystinuria and cystine stone formation. Note the great excess of cystine (1), arginine (14) and lysine + ornithine (which overlap) (13)

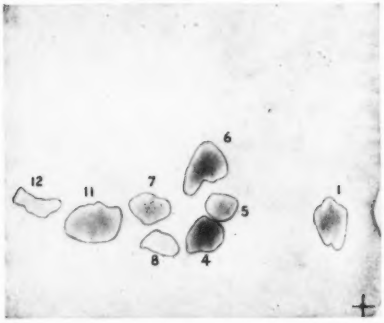

FIG. H. Urine from a patient with hepatic cirrhosis. Note the excess of cystine, taurine and β-amino*iso*butyric acid when compared with normal urine (Plate I, fig. D)

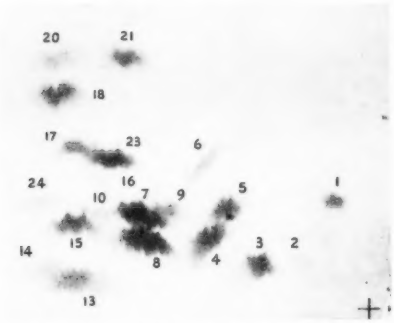

FIG. J. Plasma from a patient with massive hepatic necrosis, 625 μl. of ultrafiltrate. Note particularly that, when compared with normal plasma (Plate I, fig. B), the concentrations of methionine (oxidized to the sulphone, 23), cystine (oxidized to cysteic acid, 1), tyrosine (21) and phenylalanine (20), also γ-aminobutyric acid (15) and glutamic acid (3), are raised

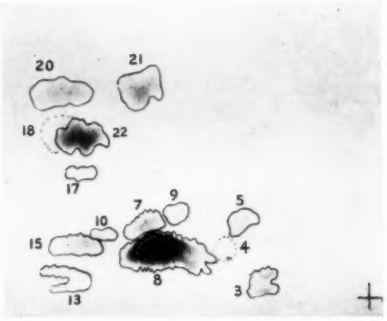

FIG. K. Cerebrospinal fluid from a patient with massive hepatic necrosis, 625 μl. of ultrafiltrate. Note that, when compared with normal cerebrospinal fluid (Plate I, fig. C), all the amino acids are present in higher concentration and that there are particularly high concentrations of glutamine (8) and methionine (22). The latter amino acid is not detected in 625 μl. of normal cerebrospinal fluid

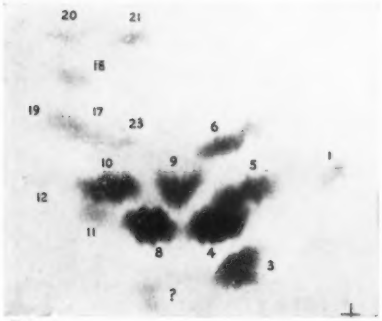

FIG. L

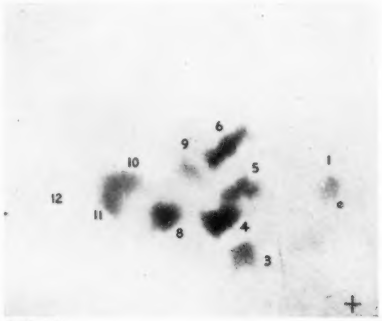

FIG. M

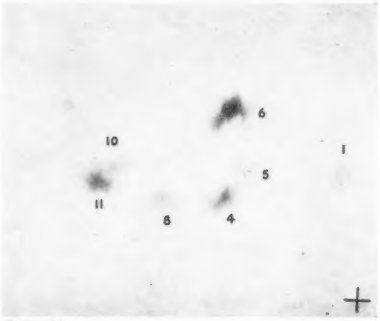

FIG. N

FIGS. L—N. Three specimens of urine, collected at 12-hr. intervals, from a patient with massive hepatic necrosis during the stage of recovery. In the first (fig. L) there is a very gross aminoaciduria. In the second (fig. M) the pattern and concentration of amino acids are still abnormal; of particular interest is the very low concentration of alanine in the presence of a high glutamine (8) concentration. In the third specimen (fig. N) the picture is now almost normal. The patient went on to a complete recovery

1: cysteic acid (from cystine); 2: aspartic acid; 3: glutamic acid; 4: glycine; 5: serine; 6: taurine; 7: alanine; 8: glutamine; 9: threonine; 10: histidine; 11: β-amino*iso*butyric acid; 12: methylhistidine; 13: lysine; 14: arginine; 15: γ-aminobutyric acid; 16: α-amino-*n*-butyric acid; 17: valine; 18: leucine or isoleucine; 19: ethanolamine; 20: phenylalanine; 20A: tryptophan; 21: tyrosine; 22: methionine; 23: methionine-sulphone; 24: proline; 25: citrulline; 26: phosphoethanolamine; 27: "cat spot" (felinine); 28: artifact due to chloride; 29: artifact due to protein

Book Reviews

Practical Chromatography

R. C. Brimley & F. C. Barrett. London: Chapman & Hall Ltd., 1953. 128 pages; 34 figures. 22 × 14 cm. 15s.

There is no doubt that, amongst the many books being published on chromatography, there is a demand for a modest handbook on the practical aspects of the subject. The present authors have set out to fulfil this demand and few will disagree with the general lay-out of the book. There is a short introductory chapter on the different types of chromatography and the theoretical considerations on which they are based. There follow two chapters on paper chromatography, and three on column chromatography—divided into adsorption, partition and ion exchange. The book ends with a chapter on accessory apparatus—fraction cutters, de-salters, and so on. There is a short appendix of solvents, sprays and R_F values, and a brief list of references.

The book would have been much clearer as a practical handbook if it had been less historical. There seems little point in giving a summary of the original paper of Consden, Gordon and Martin (published in 1944) or in describing the many improvements in the preparation of silica gel; far better for the authors to describe how they would do it now after years of experience.

The chapters on paper chromatography will be found helpful but the relative merits of the ascending and descending methods should have been pointed out. The choice of paper is also briefly dismissed with an inadequate table.

To deal briefly with all the different types of adsorption chromatography on columns is difficult, but from the severely practical point of view this chapter is too long. The gas–liquid partition system devised by James and Martin is dealt with fully in the chapter on partition chromatography on columns. This is very welcome, for it is undoubtedly a method with a very wide application and is not, at present, very well known. Craig's countercurrent method is very summarily dealt with in the last paragraph of this chapter. The impression is given that this is a pretty poor method—which it most certainly is not.

While a book of this nature must necessarily have a bias towards the methods with which the authors are familiar, it is a pity that the opportunity of a balanced account of ion-exchange chromatography has been missed. Apart from the fact that in the chapter on this subject there is practically no mention of any substance not occurring in a protein hydrolysate, the displacement methods of Partridge and his co-workers, excellent though they may be, occupy far too much of the space available. The resolving power of the elution technique of Moore and Stein for the separation of amino acids is much greater than that of any other published method and should have received more attention. Furthermore, there is no mention of the most excellent preparative methods in which hydrochloric acid or volatile buffers are used to elute amino acids from a column of Dowex 50. In this chapter too, the details of the different ion-exchange resins available and their elementary chemistry are not dealt with adequately.

In spite of the above criticisms, this book is very welcome and will undoubtedly be found most useful. It has obviously been carefully written and is well produced at a reasonable price.

P. N. Campbell

Chromatographic Methods of Inorganic Analysis, with Special Reference to Paper Chromatography

F. H. Pollard & J. F. W. McOmie. London: Butterworths Scientific Publications, 1953. viii + 192 pages; 1 plate; 26 figures. 22 × 14 cm. £1 10s.

The authors of this book have succeeded in producing an excellent account of a modern technique of analysis—one that has already proved invaluable in many instances. This volume, devoted solely to inorganic analysis, covers extensively the use of filter-paper and cellulose pulp as adsorbents, but a chapter deals with the use of alumina, silica gel, quartz and other adsorbents.

The history of the subject, and a simple non-mathematical treatment of the theory of adsorption and partition chromatography, which has not kept pace with practice, are dealt with in the first chapters. A description of the techniques for the preparation of chromatograms is followed by an account of the location and identification of zones. The many factors influencing the choice of solvent for the separation of cations, the effect of complexing agents, of water, and of acidity, the interference between cations, and that of anions, time, temperature and chemical equilibrium effects are discussed. A table of some hundred solvent mixtures used for the separation of cations forms a very valuable appendix, and doubtless contains the answers to many problems not yet posed.

Two schemes are proposed for the qualitative analysis of unknown mixtures of cations: the differential spraying method, and the chromatogram-comparison method, applicable to some twenty common cations. Chapters are devoted to the separation of anions, and to quantitative separations and estimations, using both paper strips and cellulose columns, in some cases in conjunction with conventional techniques of analysis. The final chapter serves as an introduction to electromigration and electrochromatography.

Unfortunately the scope of the book was not intended to cover the application of ion-exchange resins.

Throughout, adequate practical details of the procedures are given, and the text is suitably illustrated. Each chapter has a full bibliography and there is a good index. The book is recommended as of value to analysts and others interested in this fascinating branch of chemistry.

A. A. North

Isotopic Tracers: a Theoretical and Practical Manual for Biological Students and Research Workers

G. E. Francis, W. Mulligan & A. Wormall. London: Athlone Press, 1954. xvi + 306 pages; 51 figures. 22 × 14 cm. £1 17s. 6d.

Isotopes have now become recognized tools in most biochemical laboratories. Like most new tools they have been used (or misused) as much for their novelty appeal as for their true value. It is therefore of first importance that a new book on the subject should present the technique in proper perspective with due consideration for its limitations as well as its advantages. No doubt the excellent way in which this is done in *Isotopic tracers* arises from the fact that the authors are experienced teachers of the subject, having conducted a practical course of instruction and lecturing in it at St Bartholomew's Hospital Medical School, London, for several years.

The book is essentially a practical manual arising out of this training course and assumes no previous experience on the part of the reader. It may appear elementary to some, but most will greatly appreciate its uncomplicated style and clear explanations of physical principles. It is clearly illustrated and is accompanied by well-chosen references for further reading at the end of each chapter. The process descriptions are convincing because they are written by people clearly accustomed to carry out the manipulations themselves. The book undoubtedly fills a gap in the available range of textbooks and can be heartily recommended to biologists wishing to make use of tracers for the first time.

In spite of all its good points there are shortcomings which will no doubt be put right in later editions. For instance, biological

labelling is said to be inferior to chemical labelling on the ground that it gives products of lower specific activity, whereas recent developments with yeasts and algae have produced a range of ^{14}C compounds of a higher order of specific activity than were hitherto available by chemical methods. A great deal of space is devoted to the mass spectrometer (manufactured by the Consolidated Engineering Corporation, USA)—an expensive instrument which is to be found possibly uniquely in Great Britain at St Bartholomew's Hospital. It receives four illustrations while there are none for the much simpler falling-drop method of assaying deuterium. While the section on counting methods is generally excellent, in some things it is not quite up to date. The Lauritsen electroscope is seldom used now and decade scalers have practically ousted the earlier scale-of-64 instruments, while the still more recent decatrons are not mentioned. A too-gloomy picture is painted of the electronic complexity of scintillation circuits and the chapter on autoradiography must be unique in having no illustrations.

It is gratifying to be able to say that the all-important subject of protection from the harmful effects of radiation is most adequately dealt with. The general procedure given for tracer experiments is admirably designed to avoid spurious results arising from laboratory contamination. On the other hand it is surprising to read that a ^{131}I solution should be poured drop by drop and the tube thrown " into the radioactive waste bin without allowing it to touch anything else "—the iodinated product being described later as " highly radioactive ".

Chromatographic procedures are referred to at intervals and full details are given for the chromatographic identification of iodine-labelled substances in urine.

A. S. McFarlane

Biochemistry in Relation to Medicine

C. W. Carter & R. H. S. Thompson. 2nd ed. London : Longmans, Green & Co., 1953. xi + 524 pages ; 1 plate ; 36 figures. 22 × 14 cm. £1 10s.

The publication of a second edition of this book only four years after its first appearance is an indication of its success, apart from its being necessitated by the rapid progress in recent years in biochemistry. The principal aim of the book, i.e. the presentation of those aspects of biochemistry that are significant to medicine, has been retained, but new chapters on lipid and protein metabolism have been added and several chapters entirely rewritten. The chemistry of the body constituents is presented in the first 89 pages in a clear, easily assimilable form; the remainder of the book is devoted to the presentation of the dynamics of the various bodily reactions, or generally to the many aspects of metabolism.

The great progress achieved in medical treatment during the last quarter of a century through advances in biochemistry is a convincing proof of the value of the scientific as opposed to the empirical approach to medicine, and therefore the medical student must necessarily equip himself with the new knowledge. Of course there are many problems in biochemistry which may not have an immediate bearing on medical practice, yet their understanding is of paramount importance for a proper appreciation of life processes. Some of these, such as mechanisms of enzyme action, and biological oxidations, are also included in the book and rightly emphasized by the authors. Particularly the exposition of the processes of biological oxidation excites admiration, although other chapters are of equally high standard.

A sound judgement enabled the authors to keep a good balance between well-established facts and mere hypotheses, and consequently there are but few statements which will have to be altered in further editions in the light of recent developments since the present text was written.

The practical examples given at the end of each chapter are based on long-standing experience.

Not only medical students but teachers of medical biochemistry and all concerned with the scientific basis of medicine will find this book valuable. The publishers are also to be commended for the careful production.

G. Popják

Biochemical Investigations in Diagnosis and Treatment

John D. N. Nabarro. London : H. K. Lewis & Co. Ltd., 1954. ix + 299 pages ; 5 figures. 22 × 14 cm. £1 5s.

This book is intended for " hospital residents and registrars ", the author informs the reader in his preface. The intention is clearly to provide for this particular section of young clinicians—much concerned in hospital practice with the " prescribing " of laboratory investigations—a guide for intelligent " prescribing " and for interpretation of the results of the tests.

The book is divided into fourteen chapters: the first four are devoted to disturbances of water and electrolyte metabolism and acid-base equilibrium. Subsequent chapters deal in turn with protein, carbohydrate and fat metabolism, with diseases of the gastrointestinal tract, liver and kidney, with the testing of the cerebrospinal fluid, with endocrine disorders, with vitamin deficiencies and lastly with some of the commoner poisonings. The book concludes with tables showing the normal values for the contents of various body fluids and excreta. These tables are, somewhat arbitrarily, divided into two sets, one under the heading: " Investigations usually undertaken in Hospital Laboratories " and another under the title: " Investigations not usually undertaken in Hospital Laboratories ". May not such discrimination invoke the idea that the investigations falling in the latter group are of no importance?

Any adverse criticism that is made of this book is due to the reviewer's impression that the author aimed too low rather than too high. Although in each chapter and their various subdivisions an attempt is made to describe briefly the biochemical basis of the metabolic disorders, nevertheless this is given in such elementary terms and so briefly that it is unlikely to satisfy the serious student of clinical biochemistry. It is rather remarkable that a book on biochemical investigations can be written without the use of chemical formulae of at least the simpler body constituents, carbonic acid and bicarbonate being the only exceptions!

On the other hand the value of the various biochemical determinations on body fluids and excreta for diagnostic purposes and for evaluation of treatment are set out clearly and in detail. The emphasis throughout is on investigations that may give the most useful information in clinical practice. As the book is not meant for clinical biochemists the practical details of the various tests are given only when the clinician might himself perform these with simple reagents, almost at the bedside.

In short, if the reader wishes to have advice whether a glucose tolerance test should be done in a particular case or not, or how to control an ion-exchange resin therapy, but is relatively unmoved by the biochemical mechanism of ketone body formation, this book will be found useful.

G. Popják

British Standard 1991. Part I. General : Letter Symbols, Signs and Abbreviations

British Standards Institution. London: British Standards Institution, 1954. 45 pages. 21 × 14 cm. 6s.

This British Standard (B.S. 1991, Part I) is the result of combining B.S. 580: 1934, *Engineering symbols and abbreviations*, and B.S. Standard 813: 1938, *Chemical abbreviations and symbols*, especially in so far as these related to the more fundamental quantities of common interest to physicists, chemists and engineers. It is appreciated that alternative uses for symbols are necessary, even in a restricted field of science, but the advantages of general use of agreed symbols for the fundamental physical quantities are urged. Account has been taken of the latest reports of relevant national and international bodies, including those referring to current usages of engineering symbols in the USA and other countries. Practically complete agreement has been reached by chemists and physicists over a wide range, although it is clear that, owing to differences of language, international agreement over the abbreviations of words is not to be expected. B.S. 1991 comprises three sections. The first is on general principles; the second covers specific usages of signs, symbols and abbreviations denoting mathematical operations and constants, symbols for physical quantities and chemical elements, and abbreviations for units; the third is a section of indexes. This publication will be of use to workers in practically any branch of science and technology.

O. M. H.

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